Cystic Fibrosis Pseudomonas Opsonins

INHIBITORY NATURE IN AN IN VITRO PHAGOCYTIC ASSAY

ROBERT B. FICK, Jr., GARY P. NAEGEL, RICHARD A. MATTHAY, and HERBERT Y. REYNOLDS, *Pulmonary Section, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510*

ABSTRACT *Pseudomonas aeruginosa* infection plays a primary pathogenetic role in the chronic respiratory tract disease of cystic fibrosis (CF) patients. Despite pronounced humoral immune responses, reflected by high levels of antibodies against Pseudomonas in serum and in sputum, the antibodies do not eliminate this bacterium. In the present study we have used affinity chromatography with a lipopolysaccharide substituted immunoadsorbent gel to isolate high titers ($mean_{CF}$ = 1:256) of immunotype specific Pseudomonas IgG antibodies from the sera of nine CF subjects, and have evaluated the functional ability of these antibodies to promote phagocytosis and intracellular killing of *P. aeruginosa* in an in vitro human alveolar macrophage culture system.

The phagocytic and intracellular bactericidal kinetics revealed that CF IgG antibodies function in an inhibitory fashion. Both the rate of phagocytosis ($rate_{CF}$ = 204 cpm/unit time) and absolute bacterial uptakes maximal at 120 min ($uptake_{CF} = 18 \times 10^3$ ^{14}C cpm) were inhibited compared with appropriate positive controls (hyperimmune serum, HIS; [$rate_{HIS}$ = 399; $uptake_{HIS} = 29 \times 10^3$, $P < 0.005$]). The ability of such CF-derived opsonins to potentiate macrophage intracellular bactericidal processes was mildly impaired (bacterial $survival_{CF} = 15 \times 10^3$ colony forming units (CFU)/min, $survival_{HIS} = 9 \times 10^3$). Further characterization of this defect, assessed with functional studies of the Fab and Fc portions of the immunoglobulin molecule, revealed an impairment in the attachment of these specific antibodies to the alveolar macrophage membrane Fcγ receptors. Preliminary studies of the physical-chemical properties of these immunoglobulins were normal. The expression of this inhibitory activity in vivo may facilitate Pseudomonas colonization and the subsequent established infections in the respiratory tracts of CF subjects.

INTRODUCTION

Chronic, relentless pulmonary infection develops in the vast majority of people with cystic fibrosis (CF),[1] frequently dominating the clinical picture, and determining the fate of the majority of them. 70–90% of CF patients are chronically colonized in the respiratory tract with *Pseudomonas aeruginosa* (Pseudomonas), and >90% die of progressive pulmonary insufficiency complicated by infection with the same bacterium (1). Pulmonary infections in CF are often associated with a distinctive and predictable bacterial etiology, the mucoid colonial form of Pseudomonas. The mucoid CF strain has a reproducible serotyping pattern that is often immunotypes 4, 6 or type 8 when the Fisher serotyping and Homma schemes, respectively, are applied to clinical isolates (2). At present, the propensity for Pseudomonas to infect the respiratory tract of CF patients is inadequately explained.

This work was presented in part at the American Thoracic Society Meeting, 18–21 May 1980 and appeared in abstract form in 1980. *Am. Rev. Respir. Dis.* **121**: 68.

Dr. Fick is the recipient of a Francis B. Parker Foundation Fellowship.

Address reprint requests to Dr. Robert B. Fick, Jr., LCI 109, Pulmonary Section New Haven, Connecticut 06510.

Received for publication 18 June 1981 and in revised form 24 June 1981.

[1] *Abbreviations used in this paper*: AM, alveolar macrophages; CF, cystic fibrosis; CFU, colony-forming unit; CML, chronic myelocytic leukemia; COLD, chronic obstructive lung disease; FITC, fluorescein isothiocyanate; F-Ps, fluoresceinated Pseudomonas immunotype 4; GVBS refers to a standard solution of barbital buffered NaCl supplemented with 1 M $MgCl_2 \cdot 6H_2O$, 0.3 M $CaCl_2 \cdot 2H_2O$, and 0.1% gelatin; HA, hemagglutination; HIS, hyperimmune serum; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; PMN, polymorphonuclear granulocyte; Pseduomonas referes to the bacterium *Pseudomonas aeruginosa*; Pseudomonas antibodies refers to particular *Pseudomonas aeruginosa* lipopolysaccharide type specific IgG opsonins; SPA, *Staphylococcus aureus* protein A; IIa, the compound, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril.

The cellular and humoral immune systems in subjects with CF appear to function normally (1, 3–5) with one major exception: whole serum appears to interfere with the phagocytic function of alveolar macrophages (AM). Whole serum from homozygous CF patients inhibits the ability of rabbit and human AM to ingest and destroy Pseudomonas bacteria (6–9). This effect has been variously attributed to a serum deficiency, to a heat-labile factor, or more recently, to a heat-stable inhibitory factor in CF sera (6–9). "Bactericidal blocking" antibodies have been suggested as an explanation for the selective inability of CF whole serum to support Pseudomonas bactericidal activity (10).

Optimal phagocytosis of Pseudomonas by polymorphonuclear leukocytes (PMN) or AM requires the presence of heat-stable opsonins (11, 12), and of the heat-stable opsonins IgG has superior protective activity when compared with secretory IgA or IgM (12–14). Murphy et al. (15) have studied the role of complement in the phagocytosis of Pseudomonas by rabbit AM. These authors concluded that specific antibody is required for efficient phagocytosis but that in the presence of an excess amount of antibody (as in CF) complement was not essential to maximize phagocytosis. Similarly, IgG antibodies in convalescent sera have been shown to augment phagocytosis of Pseudomonas even in the absence of heat-labile (complement) opsonins (16, 17). Because of the importance of immune IgG in the phagocytic process, and special constraints in antibody binding to macrophages imposed by specific cell surface receptors (18), it is likely that such an opsonic antibody deficiency would be in the IgG class.

While CF patients have a generous antibody response in serum, sputum, and to a lesser degree, in lung lavage fluid to *P. aeruginosa* somatic antigens, the functional capacity of these specifically purified antibodies has not been studied directly. To investigate the opsonic, phagocytic, and bactericidal properties of *P. aeruginosa* lipopolysaccharide type-specific IgG opsonins (Pseudomonas antibodies), we used affinity chromatography to isolate antibody from CF sera with natural affinity for Pseudomonas lipopolysaccharide (LPS) antigens. After isolation we examined the immunological, functional, and physicochemical characteristics of these CF antibodies, attempting to explain the inhibitory nature of CF opsonins and thus provide alternative methods for improving pulmonary host defenses.

METHODS

Serum specimens and isolation of IgG. Peripheral blood was obtained from nine patients (seven males, two females) with an established diagnosis of CF (mean age 20 yr, range 17–31), and from one patient with intrinsic asthma and chronic bronchitis (COLD). These subjects were clinically stable but had experienced numerous respiratory infections with *P. aeruginosa*, and their sputum remained colonized with Pseudomonas organisms. Blood from actively immunized subjects included pooled serum from normal volunteers (hyperimmune serum, HIS) and individual sera from three patients with chronic myelocytic leukemia (CML) in remission; all were immunized with a heptavalent Pseudomonas lipopolysaccharide vaccine (19). Serum from a normal volunteer who had no history of a previous Pseudomonas infection and had not been immunized served as a negative control. Serum was preserved at −35°C until assayed.

To obtain an IgG-enriched globulin fraction (20), serum was precipitated with 30% ammonium sulfate. The precipitate was dissolved in borate-saline buffer (0.2 M H_3BO_3 and 0.16 M NaCl, pH 8.0) and dialyzed extensively until sulfate ions were no longer detected in the dialysate. A Sephadex G-200 gel (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) column (2.6 cm [i.d.] × 100 cm) was prepared in borate-saline buffer, and the void volume determined with 5.0% Dextran Blue 2000 (2×10^6 daltons, Pharmacia). A sample of the IgG-enriched fraction, equal to 2–3% of gel bed volume, was applied to the top of the gel bed and the flow rate was adjusted to 10 ml/h (Holter roller pump, model 907, Extracorporeal Medical Specialties, Inc., King of Prussia, Pa.). Eluate fractions of 5 ml were collected and the elution position of IgG was identified with rabbit antiserum specific for the heavy chain of IgG by double radial immunodiffusion precipitation.

Preparation of immunoadsorbent. Monovalent *P. aeruginosa* somatic antigens, Fisher immunotypes one to seven, were provided by C. L. Heifetz, Warner-Lambert/Parke-Davis, Pharmaceutical Research Division, Detroit, Mich. These complex LPS antigens contained 2.7–7.6% protein as measured by the Lowry method, and 15–32% carbohydrate as determined with a phenol-sulphuric acid reagent (21). Pseudomonas LPS immunotype 4 was radioiodinated by modifying the method of Fraker and Speck (22) and the LPS was used in preparation of an affinity gel. 4 μg of 1,3,4,6-tetrachloro-3a, 6a-diphenylglycoluril ("IIa," Pierce Chemical Co., Rockford, Ill.) and 20-μl aliquots of methylene chloride were dispensed into 10 × 75-mm borosilicate glass tubes. The tubes were placed in a 37°C water bath, and rotated by hand until the methylene chloride evaporated. These IIa-coated tubes were stored in a desiccator and protected from light. For iodination of type-specific LPS, 50 μl of 4.8 mg/ml LPS stock suspension; 5 μl of KI, as a 2.2 μg/ml solution; and 5 μl of ^{125}I, as NaI^{125} 5 mCi/50 μl solution (New England Nuclear, Boston, Mass.), were added to a IIa-coated glass tube. The final volume in the tube was adjusted to 100 μl with phosphate-buffered saline, pH 6.8; then the tube was rotated by hand on crushed ice for 20 min. and transferred to an uncoated tube. Gel filtration (Sephadex G25 Coarse, Pharmacia) was used to remove unbound NaI^{125}. Labeling of the complex bacterial cell wall LPS achieved an activity for the type 4 LPS-I^{125} of 2.3–4.4 × 10^6 cpm/mg which allowed quantitation of the percent LPS bound to the Sepharose gel matrix.

The procedure for coupling type 4 Pseudomonas LPS to Sepharose gel, detailed previously (23), can be summarized as follows: CNBr-activated Sepharose 4B (Pharmacia) was added to a coupling solution of 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.0, with 20 mg of LPS. The type-specific LPS had been mixed with a trace amount of ^{125}I-labeled LPS obtained by radiochromatography. The mixture was tumbled, excess (unbound) LPS was removed, and the remaining active groups on the gel were blocked. A 0.5-ml aliquot of the final affinity gel and aliquots from each wash

solution were counted to determine the fraction of labeled LPS which bound. The affinity gel was stored at 4°C in the borate salt working buffer.

The IgG-rich fractions obtained previously from CF and various immune or control sera were added to the LPS affinity gel. Bound antibodies were eluted with 0.05 M glycine-HCl buffer pH 2.4 and were collected in a flask containing 5% NH_4HCO_3 solution. The concentrations of both the eluted Pseudomonas-specific antibodies and the nonadsorbed antibodies were determined by radial immunodiffusion (Tri-Partigen RID plates, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) or by a quantitative immunofluorescent assay (Bio-Rad Laboratories, Richmond, Calif.). Antibody activity against the individual Pseudomonas immunotypes was screened by a slide agglutination method (2), by double radial immunodiffusion precipitation tests performed in 1.2% Noble Agar (Difco Laboratories, Detroit, Mich.) in borate-buffered saline, and quantitated by a passive microhemagglutination (HA) assay. Additionally, we performed microimmunoelectrophoresis, using 1.2% agar ("low ion," Difco Laboratories) in 0.03 M barbital buffer to test antibody purity.

Lung lavage and establishment of short-term in vitro cell cultures. Informed consent for lung lavage was obtained from 15 normal, healthy adult volunteers. The subjects, both smokers and nonsmokers, had no history of a recent acute respiratory illness nor chronic pulmonary disease. Transnasal fiberoptic bronchoscopy (model 5 BF2, Olympus Corporation of America, Hyde Park, N. Y.) was performed using a previously described method (24). For subsegmental lavage, 200–300 ml of sterile normal saline in 50-ml aliquots were instilled into the lingula and then into the right middle lobe and aspirated by syringe. The lavage fluid return was 67%±10 (mean±SD). Lavage fluid was centrifuged at 500 *g* for 10 min at 4°C to sediment the respiratory cells. The supernatant fluid was decanted and the cell pellet was washed twice in modified Hanks' balanced salt solution (Ca^{++} and Mg^{++} free, Grand Island Biological Co., Grand Island, N. Y.). The respiratory cell pellet was resuspended in serum-free McCoy's 5A medium (Grand Island Biological Co.) supplemented with 100 U penicillin and 100 μg streptomycin. A cell count by hemocytometer, viability by trypan blue dye exclusion, and differential count of Wright's-stained cytocentrifuge-prepared cells were performed (25). The cells were then adjusted to a concentration of 1×10^6 viable cells/ml in the antibiotic supplemented McCoy's 5A medium; 1 ml was placed into 4 cm^2-glass-bottom tissue culture chambers (Lab-Tek Products, Div. Miles Laboratories, Inc., Naperville, Ill.) and incubated 16 h in an humidified air and 5% CO_2 atmosphere at 37°C. In the performance of a single phagocytic and bactericidal experiment we simultaneously tested the function of Pseudomonas opsonins derived from several clinical sources; however, the AM were derived from one healthy volunteer.

Radioactive bacteria. A mucoid strain of *P. aeruginosa* isolated from a patient with CF was immunotyped by R. A. Baltimore, M.D. (Yale University School of Medicine, New Haven, Conn.). To prepare a stock culture, these immunotype 4 reference organisms were grown in a humidified air and 5% CO_2 environment at 37°C on tryptic soy agar (Pfizer Diagnostics Division, Pfizer, Inc., New York) for 20 h. Individual colonies were harvested, distributed into 5-ml vials of 20% (vol/vol) sterile glycerol-enriched tryptic soy broth and frozen immediately at −70°C.

Before each phagocytic assay, a vial of the type 4 Pseudomonas was thawed and inoculated into a 125-ml Erlenmeyer flask containing 50 ml of tryptic soy broth and 0.05 mCi ^{14}C-L-amino acid mixture (New England Nuclear). The culture media was rotated for 16 h in a humidified air and 5% CO_2 environment at 37°C. Then bacteria were sedimented by centrifugation at 2,000 *g* for 15 min and resuspended in 40 ml of sterile 0.9% saline by vigorous mixing; such washings were repeated four times until the radioactivity in the supernatant fluid was minimal. The bacterial pellet was resuspended in sterile saline to concentrations from 10^6–10^8 colony forming units (CFU)/ml by adjusting the optical density of the suspension at 620 nm; concentrations were confirmed by quantitative pour plate cultures. Throughout the phagocytic and bactericidal assays representative 100-μl samples of the original inoculum of Pseudomonas were counted to assure that the bacteria remained in lag-phase growth.

Opsonization of Pseudomonas. Pseudomonas IgG agglutinins derived by affinity chromatography were used as sources of opsonic antibodies. For opsonic experiments these antibodies were adjusted either to equititered LPS type 4 HA levels, when derived from immunized subjects and those with chronic Pseudomonas infections; or to equivalent milligram per milliliter protein concentrations when the immunoglobulins were derived from negative controls. Washed suspensions of ^{14}C-labeled Pseudomonas organisms in saline (10^8/ml) were added to equal volumes of the Pseudomonas antibodies and incubated at 38°C for 30 min. During the incubation of Pseudomonas with the IgG opsonins, samples were examined periodically using phase-contrast microscopy (Invertoscope, D. Zeiss, Oberkochen, West Germany) and incubation times were limited so that visible aggregation of bacteria would not occur. These opsonized and washed bacteria in 200-μl aliquots were dispensed into the human AM monolayer cultures. Pseudomonas antisera from four of the CF subjects were heat inactivated at 56°C for 30 min before performing the opsonophagocytic assay.

The degree of opsonization was assessed in two ways: qualitatively by using the indirect immunofluorescent method of Thomas (26) with fluorescein isothiocyanate (FITC) conjugated to rabbit anti-human IgG (Bio-Rad Laboratories) and adsorbed with type 4 Pseudomonas; and quantitatively by using ^{125}I-labeled *Staphylococcus aureus* protein A (SPA). The Iodogen method (22) proved effective for labeling SPA, achieving an activity for the ^{125}I-SPA of 3.6–6.9×10^8 cpm/mg. The iodinated SPA eluted as a sharp peak from a Sephadex G-25 column and was easily separated from the unbound NaI. To test for the degree of antibody coating, aliquots of opsonized Pseudomonas type 4 were centrifuged at 2,000 *g* for 15 min, the supernatant fluid containing excess IgG was decanted, and the bacterial pellet washed in 3 ml of 0.9% NaCl. SPA with a binding capacity of 9 mg human IgG/mg (Sigma Chemical Company, St. Louis, Mo.) was labeled with ^{125}I by the Iodogen method of Fraker and Speck (22). Excess radioactive SPA, determined by a quantitative agglutination reaction in a modified Farr test (27), was added to the Pseudomonas-opsonin pellet and incubated at 37°C for 60 min with occasional vortexing to disperse the mixture. The bacteria-IgG-SPA complexes were centrifuged at 2,000 *g* × 15 min and washed twice with 0.9% NaCl before counting duplicate specimens in a gamma counter. Opsonization, as well as the phagocytic and bactericidal assays, were performed using both saline and HA negative IgG for controls.

Pseudomonas phagocytic assay. The ^{14}C-labeled Pseudomonas immunotype 4 uptake by human AM was evaluated with a phagocytic assay used by Reynolds and Thompson (12). In brief, the adherent macrophage monolayers were washed twice in Hanks' balanced salt solution decanted, reconstituted to a volume of 1.8 ml of Hanks' balanced salt solution and allowed to acclimate in a humidified environment containing air and 5% CO_2 at 37°C for 15 min.

200 μl of opsonized radiolabeled Pseudomonas was added to the macrophage monolayers (10:1 ratio of bacteria: macrophages), and the tissue culture chambers were reincubated at 37°C with continuous rotation. As a further test of cell viability during the assay, duplicate monolayers were selected and the cytosolic enzyme lactate dehydrogenase (LDH) was measured in aliquots of the culture medium and monolayer homogenate (28). At predetermined intervals chambers were selected, the supernatant fluids were decanted and the cell layers washed three times with 2 ml of sterile Hanks' balanced salt solution. The cell layers were disrupted by the addition of 2 ml of distilled water, scraped with a rubber policeman, and examined using phase-contrast microscopy to assure complete lysis. A 100-μl aliquot of this homogenate was used to determine intracellular bacterial survival and to calculate bactericidal rates as detailed below.

To determine the phagocytic rates, supernatant fluids, cell washes and all but 100 μl of the cell lysates were transferred to individual 17 × 58-mm scintillation vials and dried in an 80°C water bath under a stream of nitrogen on an analytical evaporator. The dried supernatant fluids and cell lysates were digested at 37°C for 12 h with 0.4 ml of a tissue solubilizer (Protosol, New England Nuclear) and 25 μl distilled water. After adding 5.5 ml of a premixed scintillation solution (Econofluor, New England Nuclear) and vortexing vigorously, the vials were counted for ^{14}C activity in a liquid scintillation spectrometer (LS-100C, Beckman Instruments, Inc., Fullerton, Calif.). The counting efficiency of this scintillation counter was monitored with ^{14}C radioactivity standards (New England Nuclear). The scintillation vials were counted twice for 10 min at a time and the net counts expressed as counts per minute. The percent of the total bacterial inoculum that was associated with the monolayer or "phagocytosed" at a particular sampling time (percent uptake) was calculated using the formula: % uptake = (^{14}C cpm in monolayer lysate/^{14}C cpm in bacterial inoculum).

Duplicate AM monolayers inoculated, incubated, and then washed in a comparable manner were selected for determination of phagocytic indices. Cell chambers were air-dried, stained with Wright's stain, and 200 macrophages chosen at random from at least four oil-immersion fields (×1,000) were counted for the presence or absence of intracellular bacteria. Phagocytic indices are expressed as the percent of AM with intracellular bacilli. Those AM with engulfed bacteria were further divided into those with less than five and those with five or more organisms per cell.

Pseudomonas bactericidal assay. At various times after the opsonized bacterial challenge, the cell monolayers were carefully washed and lysed with distilled water. 100 μl of the cell homogenate was aspirated, serially diluted in sterile distilled water, and inoculated into petri dishes (100 × 15 mm) containing 10 ml of liquid tryptic soy agar. After incubation for 36 h these quantitative pour plates were examined under a dark field colony counter and the number of CFU electronically recorded. Throughout the assay representative 100-μl samples of the original inoculum of Pseudomonas were counted to assure that the bacteria remained in the lag-phase growth. The killed population of bacteria was derived according to the formulae: total number of intracellular bacteria = % uptake × CFU in original inoculum. Number of killed bacteria = uptake number − CFU in monolayer lysate.

Scanning electron microscopy. Scanning electron microscopy was used to examine the AM monolayers at varying intervals after inoculation and ingestion of Pseudomonas (29). The macrophages were washed as described for the intracellular bactericidal assay, duplicate chambers were fixed for 15 min in 1.5% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), and dehydrated in graded solutions of ethanol. Before scanning electron microscopy (ETEC, Perkin-Elmer Corp. Norwalk, Conn.), the bacteria-inoculated AM monolayers were critical point dried. In each experiment at least 50 AM were examined to derive the adherent but not internalized fraction of Pseudomonas associated with the monolayers.

AM receptor binding. The opsonin-coated Pseudomonas complexes attached to AM surface receptors were quantitated by an immunofluorescent assay. *P. aeruginosa* immunotype 4 was prepared with a direct fluorescein label by the method of Gelfand (30). Pseudomonas reference organisms (see above) were thawed and inoculated into 150 ml tryptic soy broth (Pfizer) at 37°C for 18 h, centrifuged at 2,000 *g* for 15 min, and the bacterial pellet resuspended in 5 ml 0.9% NaCl. The bacteria were heat killed at 60°C for 45 min, washed three times in normal saline and this slurry was incubated with a 0.03% solution of FITC for 2 h at room temperature. The fluoresceinated Pseudomonas organisms (F-Ps) were washed three times in a veronal-buffered saline solution supplemented with 1 M $MgCl_2 \cdot 6H_2O$, 0.3 M $CaCl_2 \cdot 2H_2O$ and 0.1% gelatin (GVBS), and stored at 70°C for later use.

Human AM were harvested, prepared, dispensed into tissue culture chambers and incubated at 37°C for 16 h as described above (24, 25). F-Ps bacteria were coated with IgG opsonins derived from CF and HIS sources, and inoculated into the washed macrophage monolayers exactly as described in the phagocytic assay earlier. The CF- and HIS-derived opsonins were used in equivalent HA titers and GVBS-fixed bacteria served as a negative control. The ratio of F-Ps to AM was constant at 10:1 for all of the assays. After challenge with opsonized bacteria, the AM monolayers were incubated at 37°C, and at various intervals the supernatant fluids from duplicate monolayers were decanted and the cell layers washed three times in GVBS. Before reading, the culture chambers were lifted from the glass slide bottom (Lab-Tek, Miles Laboratories, Inc., Naperville, Ill.) and the monolayers covered with 22-mm square glass cover slips. The total number of AM per high power field and the number with associated F-Ps were determined under visible and ultraviolet illumination, respectively. The ratio of AM binding F-Ps to total AM was determined in triplicate for the opsonin samples at each time interval after macrophage inoculation.

Physicochemical studies of IgG anti-Pseudomonas antibody. We have prepared antisera to the four human IgG γ-chain subtypes by immunizing primates (*Macaca fascicularis*) with purified IgG Fc subtype heavy chain fragments derived from a monoclonal myeloma plasma, kindly provided by Richard Wistar, M.D., Naval Medical Research Institute, Bethesda, Md. Individual antiserum specimens, adsorbed with various Fcγ fragments until monospecific, have been used in double diffusion immunoprecipitation assays to identify the γ-subclass of the Pseudomonas antibodies. Light chain subtypes were assayed with κ and λ-chain specific rabbit antisera (Behring Diagnostics, American Hoechst Corp., Somerville, N. J.).

The immunotype specific Pseudomonas IgG opsonins were layered on 10–40% linear sucrose density gradients, and subjected to density gradient ultracentrifugation at 37,000 rpm at 4° for 21 h (L5-50 ultracentrifuge, Beckman Instruments). Normal human 7S IgG labeled with ^{125}I by the methods of Fraker and Speck (22) served as a sedimentation marker.

Isoelectric focusing was performed in 250-mm thin layer 5% polyacrylamide gels (Eastman Kodak Co. Rochester, N. Y.) with carrier ampholyte gradients having a pH range of

5–8 (Pharmalyte, Pharmacia). The anode solution was 0.04 M L-glutamic acid and 0.1 M NaOH was at the cathode. The gel was prefocused with 5 W for 30 min and then electrophoresis was performed for 2 h at 30 W constant power at 7°C constant temperature. The CF and control Pseudomonas opsonins, as well as reference IgG subtype immunoglobulins, were dialyzed against distilled water before electrofocusing, and applied to the gel in equivalent quantities. The pH gradients were measured by a flat surface electrode (Orion Research, Incorp., Cambridge, Mass.), and with the assistance of pI marker proteins (Pharmacia). After fixing, the gel was stained in 0.2% Coomassie Brilliant Blue and specificity of the isoelectric bands was determined by either autoradiography using ^{125}I-LPS 4, or by LPS-coated erythrocytes (31).

Scintillation autoradiography was performed by modifying the method of Bonner and Laskey (32). 50 μl of Pseudomonas LPS 4 in a 4.8 mg/ml solution was labeled with ^{125}I, as described earlier, resulting in an activity of 1.59×10^7 cpm/mg. Before focusing, the antisera were incubated at 37°C for 30 min in the presence of this LPS antigen (10^5 cpm/IgG sample). After isoelectric focusing as detailed above, the gel was dehydrated in dimethyl sulphoxide and covered overnight with a 20% (wt/wt) solution of 2,5-diphenyloxazole in dimethyl sulphoxide. The gel, now impregnated with the scintillator 2,5-diphenyloxazole, was lyophilized at −70°C and exposed to X-Omat film (Eastman Kodak). With the use of an image intensifier screen, 30 min of exposure at room temperature was found to be optimal. The x-ray film was developed in a standard x-ray developer (Eastman Kodak).

Statistical analyses. Arithmetic means and standard deviations of the phagocytic and bactericidal data for each source of opsonin or negative control were calculated and analyzed by Student's *t* test for compared data. *P* values reported are for a two-tailed test. Slopes were calculated from the mean curves by using the method of least squares and linear regression analysis. The *r* values reported are Pearson's correlation coefficients (33).

RESULTS

Immunoadsorption and elution of type 4 Pseudomonas antibodies. HA titers of the serum specimens are given in Table I. Anti-Pseudomonas type 4 LPS titers were uniformly present and high in all but the normal (negative) control serum; titers against other somatic types were elevated as well. With type 4 LPS as a ligand and passage of the serum gamma globulin concentrates through the affinity gel, IgG antibody in high titer against type 4 *P. aeruginosa* was isolated. Other individual immunotype HA titers were measured for the specifically prepared IgG antibody specimens ("mg-ml," Table I) as well. No significant antibody reactivity (titer >4) against other LPS antigens existed in the final antibody preparations, so that further adsorption of the antibody samples was not needed to remove antibodies cross-reacting with other LPS immunotypes. As a further test of purity, the final antibody preparations were assessed for residual IgM content by reduction with 2-mercaptoethanol. There were no demonstrable changes in the HA titers before and after reduction. Microimmunoelectrophoresis of all the Pseudomonas specific antisera showed a single precipitin arc against anti-whole serum antibody.

Functional assays of Pseudomonas opsonins with

TABLE I
Type-specific Anti-Pseudomonas Titers by Passive Microhemagglutination

	Before affinity chromatography							After affinity chromatography with type 4 LPS antigen						
	Reciprocal HA titers for Pseudomonas immunotypes							Reciprocal HA titers in immunoglobulin fraction Pseudomonas immunotypes						
Serum specimens	1	2	3	4	5	6	7	IgG	1	2	3	4	5	6	7
								mg/ml							
1. CF	24	256	32	1,024	16	128	64	2.6	<4	<4	<4	512	<4	<4	<4
2. CF	16	512	512	1,024	64	512	256	2.3	<4	<4	<4	512	<4	8	<4
3. CF	<4	1,024	512	1,024	64	512	128	1.9	<4	<4	8	256	4	<4	4
4. CF	<4	512	256	1,024	512	1,024	2,048	1.4	<4	<4	<4	256	8	—	—
5. CF	<4	64	4	256	4	64	8	1.3	—	4	<4	128	—	—	—
6. CF	<4	<4	64	1,280	<4	—*	<4	1.6	<4	<4	<4	64	<4	4	—
7. CF	16	8	4	64	4	16	4	1.7	4	<4	<4	32	<4	<4	<4
8. CF	<4	256	64	128	4	128	64	1.7	<4	<4	<4	64	<4	4	<4
9. CF	16	1,024	64	256	128	256	256	1.8	—	—	<4	256	8	—	—
10. COLD	<4	128	64	128	8	256	32	1.4	4	4	4	64	<4	8	<4
11. CML	—	—	64	64	—	1,024	32	0.8	<4	—	16	16	<4	8	—
12. CML	—	—	32	128	—	—	—	1.3	<4	8	<4	64	—	<4	—
13. CML	<4	—	<4	1,024	—	32	<4	0.9	<4	4	<4	64	<4	<4	—
14. Hyperimmunized normal	128	1,024	2,048	4,096	512	512	8,192	1.7	<4	4	<4	128	<4	<4	4
15. Normal control	<4	4	<4	4	<4	4	4	0.8	<4	<4	<4	8	<4	<4	<4

* Not determined.

AM. AM, obtained from healthy tobacco smoking or nonsmoking subjects, were maintained in short-term cultures to assess the effectiveness of anti-Pseudomonas opsonins to facilitate phagocytosis and subsequent intracellular killing of a clinical isolate of viable *P. aeruginosa.* Six nonsmoking volunteers, mean age 27 yr (range 19–48), participated in the study. An average of 49×10^6 respiratory cells were recovered with mean differential cell counts of 93% AM, 6% lymphocytes, and 1% PMN. Nine smokers participated with a mean age of 33 yr (range 20–41). This group was a 26-pack/yr history of cigarette use yielded a mean of 122×10^6 respiratory cells from lavage and had mean differential cell counts of 90% AM, 7% lymphocytes, and 3% PMN. Mean cell viabilities (83%±8 SD) and the percent recoveries of lung lavage fluids (67%± 10 SD) were identical in the two groups. The macrophage yield from a single lavage provided a sufficient number of cells to assess the function of IgG opsonins derived from at least two immunized subjects and a negative control. Thus, any slight variation in AM function or in the ratio of bacteria to AM did not affect the comparison of phagocytosis in the presence of different opsonins, since the source of cells for each experiment was constant.

AM phagocytosis of opsonized Pseudomonas. Type 4 reference organisms, originally a mucoid CF clinical isolate, had been stored at −70°C in an enriched broth and were used for the opsonic, phagocytic, and bactericidal assays. Periodically, a vial of this reference Pseudomonas was studied and the bacterial serotype remained stable. Although the bacterial colonies reverted to the nonmucoid state and did not produce a pigment, they remained consistently immunotype 4. These standardized organisms grew vigorously in tryptic soy broth and readily incorporated the ^{14}C-L-amino acid label (mean CFU/^{14}C cpm = 615±100 SEM, n = 9). After washing three or four times, 98.6% ±0.7 SD of the remaining radioactivity was associated with the bacteria and the radioactivity in the supernatant fluid was minimal.

A representative experiment used alveolar macrophages from a nonsmoker, and cell monolayers (1×10^6 macrophages/culture) inoculated with 1×10^7 viable type 4 Pseudomonas opsonized with IgG from either a CF specimen (△) or from a immune normal source (○). At varying intervals after the monolayers were inoculated with opsonized bacteria, ^{14}C counts were determined in the monolayer supernatant and wash fluids, and in the cell layer homogenates. The counting efficiency of the liquid scintillation spectrometer was 90.2% and the SE of the cpm was uniformly <1.5%. Descending dashed lines of ^{14}C activity depict residual bacterial counts in supernatant media and collected in wash fluids from the monolayers (Fig. 1). These curves are essentially reciprocal values of the cell homogenate radioactivity, and their sums (dark solid lines) are equal and account for >85% of the ^{14}C cpm originally added. Recovery of radioactivity was similar within the experiment and constant in our subsequent assays.

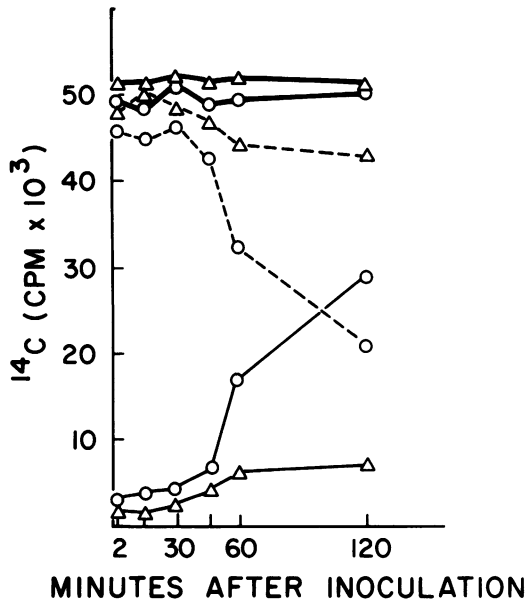

FIGURE 1 Representative phagocytic experiment. A representative experiment shows the partitioning of opsonized ^{14}C-labeled Pseudomonas organisms (ordinate) between the supernatant fluids (-----) and the homogenized AM monolayer lysates (——) at various intervals after inoculation (abscissa). The sums of radioactivity (——) from AM monolayers inoculated with HIS (○), and CF (△) IgG opsonins were equivalent.

Fig. 2 depicts composite curves for mean values found from 12 duplicate experiments in which each of the nine CF-prepared IgG opsonins (Table I) were compared with positive HIS and negative control opsonins. Repeat studies were done with CF specimens 1, 4, and 9 (Table I). Bacteria as shown by ^{14}C counts, were taken up by the cell monolayers in roughly linear fashion over the first 60 min of the assay (Fig. 2, lower panel). Linear regression analysis gave r values of 0.99 for both mean CF (△) and mean HIS (○) phagocytic curves. Despite equivalent Pseudomonas HA titers, the CF-derived opsonins (△) produced a phagocytic rate significantly less than opsonins derived from immunized normal subjects (○). Over the first 60 min of the assay the CF-opsonin-induced uptake rate of Pseudomonas ^{14}C was 204 cpm/unit time, while HIS opsonins facilitated an uptake rate of 399 ^{14}C cpm/unit time ($P < 0.005$ at 60 min). The absolute bacterial uptake, maximal at 60 and 120 min, was also much less than CF opsonins when compared to equititered HIS-derived antibodies (at both times, $P < 0.005$). The phagocytic indices (in parentheses) for the monolayers

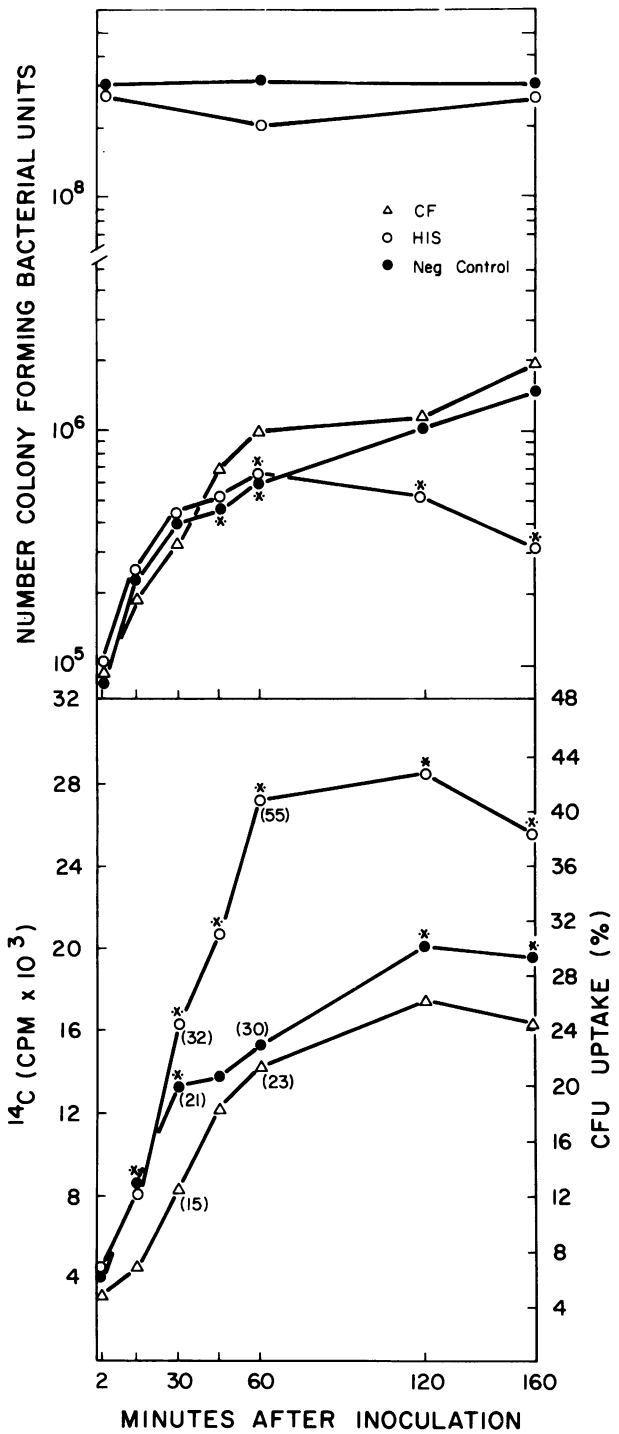

FIGURE 2 Upper panel: mean bacterial survival curves. Viable bacteria (ordinate) were grown from AM cellular homogenates. These Pseudomonas survival curves represent mean numbers of viable bacteria cultured at various time intervals (abscissa). Pseudomonas were opsonized with equal HA titers of HIS (○, 11 experiments), CF, (△, 12 experiments) IgG antibodies, and HA negative controls (●, 8 experiments). Within the first 60 min, linear regression analysis of the bacterial colony counts gave $r > 0.96$ for all three curves, and the bactericidal rates were similar. Over the last 100 min of the assay, HIS Pseudomonas opsonins promoted a cumulative bactericidal effect as the survival slope became negative and CFU decreased from 6×10^5 to 3×10^5 during this interval. The bacterial inocula incubated with the opsonin preparations are shown above the break in the ordinate's log scale. These opsonized bacteria remained in lag-phase growth over the course of the assay.

FIGURE 2 Lower panel: mean phagocytic curves. The inoculum of viable ^{14}C-labeled Pseudomonas organisms (left ordinate) and the percent uptake, expressed as a ratio of bacterial CFU (right ordinate), are shown for homogenized AM monolayer lysates, depicted at various intervals after inoculation (abscissa). The composite curves represent mean bacterial uptake of Pseudomonas opsonized with equivalent titers of CF-derived type 4 LPS-specific IgG molecules. Results from 12 experiments using CF (△) IgG antibody specimens, 14 experiments with IgG specimens from HIS (○), and 11 assays using control (●) IgG specimens without type 4 antibody activity are shown. IgG without Pseudomonas type 4 HA titers isolated from normal serum was used in protein concentrations equivalent to the CF and HIS opsonins. The mean number of AM in the monolayers having ingested bacteria (≥1) at 30 and 60 min is given in parentheses (phagocytic indices). Phagocytosis over the first 60 min of the assay was linear ($r > 0.94$ for all three opsonin sources) but the rate of bacterial uptake expressed as ^{14}C cpm/unit time was less in the presence of CF opsonins [$\text{slope}_{CF} = 204$], when compared to HIS opsonins [$\text{slope}_{HIS} = 399$]. Absolute bacterial uptakes were greater for HIS and the negative control specimens at each point in the assay and reached statistical significance ($P < 0.05$) when compared with the CF opsonins at the points indicated (*).

challenged with Pseudomonas opsonized with HIS antibodies were more than twice the indices achieved with CF opsonins (Fig. 2, lower panel).

Even more striking was the comparison of CF opsonins to negative controls (●). When compared to the negative controls (bacteria not coated with opsonic antibody), the CF bacterial uptake was less at each point in time, particularly within the first 45 min and at 120 and 160 min of the assay ($P < 0.05$). Both 0.9% saline and IgG derived from a healthy normal subject with the protein concentration adjusted to equal the protein concentrations of the CF opsonins were used as negative controls. Despite the absence of Pseudomonas HA titers, the negative controls achieved a rate of phagocytosis of 188 ^{14}C cpm/unit time, $r = 0.94$. This is compatible with earlier reports that AM are good phagocytes in the absence of any opsonins (12). In fact, the saline control opsonins achieved a phagocytic rate and absolute bacterial uptake equivalent to our nonimmune IgG fraction, and produced a greater uptake than the high-titered CF antibodies. It is remarkable that despite the absence of Pseudomonas antibody and presumably no bacterial opsonization, the uptake of nonopsonized Pseudomonas was better than CF IgG-coated bacteria. For all opsonin specimens the phagocytic indices increased over time and tended

to parallel the changes in CFU ingested (right ordinate, Fig. 2, lower panel). Heat inactivation of the opsonins at 56°C for 30 min did not alter the results. Moreover CF IgG opsonins derived from fresh, frozen, and heat-inactivated sera were compared in this phagocytic assay and gave the same results.

For contrast, Pseudomonas uptake experiments with immune opsonins prepared from three patients with CML (■) and one with COLD (□) are each compared with HIS (○) in Fig. 3. For all, bacterial uptakes were initially brisk and then rates plateaued. Equivalent titers of IgG opsonins for type 4 Pseudomonas antigen from the patient with COLD, representing chronic respiratory tract Pseudomonas colonization and natural immunization, supported a rate of phagocytosis over the first 60 min of the assay and attained a maximal phagocytic uptake that were both intermediate to HIS and negative control opsonins ($Slope_{COLD}$ = 229 cpm/unit time, $Slope_{HIS}$ = 293, and $Slope_{NEG}$ = 140; for all, $r > 0.94$). When COLD and HIS opsonins were compared, no statistically significant differences were noted in their absolute maximal uptakes. Pseudomonas IgG antibodies from CML subjects (assays, $n = 4$) immunized with the Pseudomonas heptavalent vaccine functioned poorly, despite adjustment of Pseudomonas HA titers to be equivalent with HIS and natural COLD-derived specimens (Fig. 3). The phagocytic rate was 137 ^{14}C cpm/unit time with $r = 0.96$ during the first 60 min of the assay (equivalent to $Slope_{NEG}$ = 140 cpm/unit time, despite absence of HA titers in the negative control). Over the last 100 min of the assay, IgG opsonins derived from CML subjects also produced a bacterial uptake significantly less than HIS or COLD antibodies ($P < 0.05$ at all points). However, CML opsonin-mediated macrophage phagocytosis improved slightly during the last 100 min of the assay when compared with negative controls.

In performing these experiments no significant difference was noted between the phagocytic uptake by macrophages obtained from smokers and nonsmokers. AM from tobacco smokers had no apparent enhancement or decrement in either the rate of opsonin-mediated phagocytosis or the absolute maximal uptake attained. Furthermore, in agreement with published reports (28), we could discern no differences between the viabilities of AM from smokers and nonsmokers. To assess the viability of the cell cultures, the cytosolic enzyme LDH was monitored. Measurements of the percentage of cellular LDH released into the supernate during the first 16 h in culture and then over the time-course of the phagocytic and bactericidal assays are shown for nonsmoker AM in Table II. Assuming the release of LDH reflects leakage of cytosol enzymes through disrupted membranes, we concluded that the viability of the AM remained constant during the uptake and killing assays.

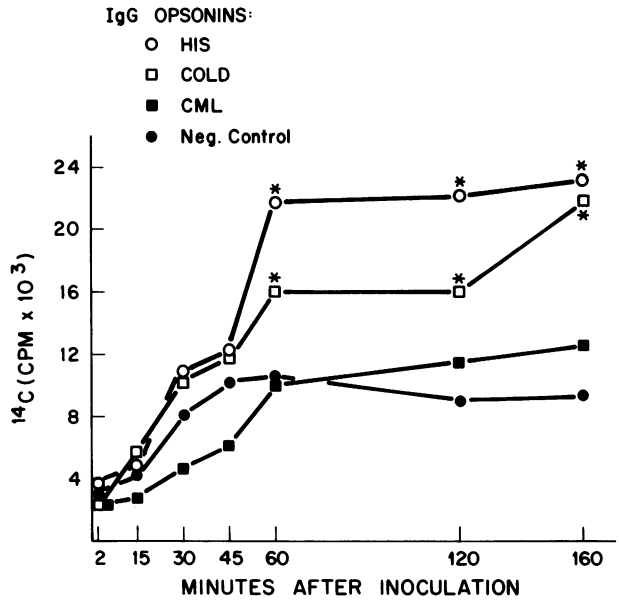

FIGURE 3 Mean phagocytic curves are shown for uptake of bacteria (ordinate) by macrophage monolayers at various time intervals (abscissa). IgG opsonins used at equivalent titers, derived from COLD (□, $n = 2$), CML (■, $n = 4$), and HIS (○, $n = 3$) sources, are contrasted. Once again the HA-negative control IgG (●, $n = 3$) was used in protein concentrations equal to that of the HA-positive opsonins. At a time when all rates of phagocytosis were linear ($r > 0.94$ over first 60 min), the naturally acquired COLD opsonins produced a rate (229 ^{14}C cpm/unit time) intermediate to those of the HIS opsonins (293) and the negative control (140). While the CML opsonins, derived from Pseudomonas LPS-vaccinated CML patients, depressed the rate of phagocytosis over the first third of the assay ($slope_{CML}$ = 137 ^{14}C cpm/unit time), maximal phagocytosis eventually did surpass that of the negative control. Statistically significant differences ($P < 0.05$) for absolute bacterial uptakes when compared to CML opsonins are indicated (*).

Pseudomonas bactericidal assay. During the above phagocytic experiments (Fig. 2, lower panel, and Fig. 3), aliquots of the macrophage monolayer lysates were

TABLE II
*LDH Enzyme Determinations**

Time	Relation to assay	$\frac{\text{LDH supernate‡}}{\text{LDH cell + supernate§}} \times 100$
h		%
0	Harvest by lavage	5
16	Beginning of assays	25
24	Assay @120 min	30
24.6	Termination	27

* Representative experiment performed on normal human nonsmoker AM (1×10^6 macrophages per culture chamber).
‡ LDH assay performed on AM monolayer supernate.
§ Assay performed upon AM supernatant and lysed AM monolayer.

serially diluted and the viable bacteria enumerated at 36 h by quantitative pour plate cultures. The number of colony forming bacteria was obtained in duplicate from two consecutive dilutions; these counts approximated each other closely. Fig. 2, upper panel, presents composite mean survival curves comparing Pseudomonas coated with CF (△, $n = 12$), HIS immune opsonins (○, $n = 11$) and the negative control IgG (●, $n = 8$). Over the first 60 min of this assay the bactericidal rates (CFU/min) were similar for all three curves ($slope_{CF} = 15 \times 10^3$ CFU/min, $slope_{HIS} = 9 \times 10^3$, $slope_{NEG} = 8 \times 10^3$); linear regression analysis of all three yielded $r > 0.96$. This suggested that initially, after ingestion had occurred, intracellular killing of the bacteria proceeded at about the same rate, irrespective of the opsonin source used. However, over the last 100 min of the bactericidal assay, the number of viable bacteria cultured from the homogenates of the cell layers exposed to the CF opsonins (△) was consistently higher than either the HA-matched normal positive (○) or negative (●) controls. Pseudomonas type 4 IgG opsonins, when derived from CF sera, did not support significant intracellular killing at later points in the assay because this bacterial survival curve rises linearly ($slope_{CF} = 6 \times 10^3$ CFU/min, $r = 0.93$) during last 100 min. Normal hyperimmune opsonins, on the other hand, facilitated a brisk intracellular bactericidal function over the last 100 min of the assay for the slope of this survival curve became negative ($slope_{HIS} = -2 \times 10^3$ CFU/min, $r = 0.90$) and was significantly lower than the CF opsonins at each point ($P < 0.02$ at each point).

To more thoroughly quantitate the number of bacteria killed at a specific point in the bactericidal assay, the number of Pseudomonads phagocytized by AM cell cultures was determined from ^{14}C cpm and then the number of viable monolayer-associated bacteria was subtracted (Table III). When AM were incubated for 120 min with pre-opsonized bacilli, 97% of the monolayer-associated bacteria were no longer viable if HIS opsonins were used; 89% were killed if originally opsonized with CF-derived antibodies. However CF opsonins promoted uptake of only 26% of the original inoculum, a percentage similar to the negative control but substantially less than achieved by HIS opsonins (42%). From the results it may be concluded that it is mainly the rate of bacterial uptake that is defective when CF opsonins are used.

To demonstrate that most of these cell-associated bacteria were in fact intracellular and not simply attached to the AM membranes, scanning electron microscopy of the cells was performed. In duplicate experiments 50 cells were examined at random from a monolayer of 10^6 nonsmoker AM after 120 min of incubation with HIS-opsonized Pseudomonas. For an AM monolayer that was not washed after incubation, the AM had a mean of 2.7 bacteria associated with each macrophage. When the usual monolayer washing procedure was performed, a mean of 1.1 bacteria (range 0–2 bacteria) were adherent to each macrophage and not internalized. Thus, routine washing of the cell layers did remove some surface adherent, non-ingested bacteria. Because 17.6 (10^6) bacteria were determined by ^{14}C cpm to be "intracellular" after carefully washing the macrophage monolayers (Table III), this represents an error of 6.2% (1.1 [10^6]/17.6 [10^6]). Similar percentages of nonspecifically adherent but not engulfed bacteria were derived when saline was used as a negative control opsonin.

The shape of the mean bacterial survival curves resulting from use of COLD and CML IgG opsonins (data not shown) resembled the HIS opsonin bactericidal curve (Fig. 2): initially increased CFU were noted and then at 60 min bactericidal activity was observed. Although the contour of these bacterial survival curves differed from the constant linear increase in CFU obtained with CF opsonins, no statistically significant differences were noted when the numbers of viable bacteria were compared from monolayers exposed to CML, COLD, and HIS opsonin sources.

TABLE III
*Opsonin-mediated Intracellular Killing of Pseudomonas after 120 min**

Opsonin source	Number of bacteria phagocytozed‡	Uptake of the original inoculum	Number of viable intracellular bacteria§	Remaining viable	Killed
		%		%	%
HIS	17.6 (10^6)	42	5.9 (10^5)	3	97
CF	10.8 (10^6)	26	1.2 (10^6)	11	89
NEG	11.9 (10^6)	28.7	9.8 (10^5)	8	92

* Phagocytosis performed at a bacteria: macrophage ratio of 10:1 with a mean inoculum of 4.2×10^7 CFU.
‡ Determined from ^{14}C cpm uptake and CFU/cpm ratios.
§ Cultured from the lysate of washed macrophage monolayers.

While the ^{14}C label and quantitative pour plate cultures permitted separate determinations of numbers of bacteria taken up and numbers killed, in an in vivo situation phagocytic and bactericidal processes would be dynamic and would occur simultaneously. This also occurred in our series of in vitro cultured cell experiments. The functional defect consistently noted in all nine CF anti-Pseudomonas opsonin preparations was striking when the phagocytic curves (Fig. 2, lower panel) and the bactericidal curves (Fig. 2, upper panel) are compared with respect to the ratio of viable CFU to ^{14}C cpm of total bacteria (Fig. 4). Despite the rapid Pseudomonas uptake achieved with HIS opsonins, the macrophage bactericidal mechanism kept pace as intracellular killing proceeded rapidly and viable intracellular bacterial counts fell over the last 100 min of the assay. This normal, intracellular accommodative mechanism with HIS opsonins (○) is reflected in Fig. 4 as the ratio (CFU/^{14} cpm) for HIS IgG is low and decreases slightly over the course of the assays. With CF opsonins (△), despite an overall poorer uptake of opsonized bacteria, there is a constant increase in the number of viable bacteria cultured from lysed cell monolayers. This is demonstrated by the increasing ratio seen at each point that is much greater than either normal hyperimmune opsonins or negative control nontitered IgG (●). Thus, AM bactericidal function fails to keep pace in the presence of CF IgG opsonins. These intracellular bactericidal kinetics were not altered when smoker AM were compared with nonsmoker AM. In fact, one smoker was lavaged twice providing AM monolayers that produced similar opsonin-mediated phagocytic and bactericidal results each time.

Physicochemical properties of Pseudomonas IgG opsonins. Initially, in preparing CF, normal hyperimmune, and negative control opsonins, sera were gel filtered through a Sephadex G200 column (exclusion limit 6×10^5 daltons) and the IgG fractions containing the Pseudomonas opsonins eluted cleanly and reproducibly at 49–57% of gel bed volume, irrespective of the serum source. After passage through the type 4 affinity column, the Pseudomonas IgG antibodies, whether obtained from CF or control sera, reacted with both κ- and λ-light chain specific antisera. Similarly, these IgG antibodies were heterogeneous with respect to the heavy chain subtypes, as all four subtypes were present and there did not appear to be a significant difference in the relative proportion of IgG subtypes when the opsonins were analyzed semiquantitatively using human IgG γ-chain subtype specific antisera.

The Pseudomonas IgG antibody samples were examined further for sedimentation characteristics by ultracentrifugation in sucrose density gradients. The radioactivity of the ^{125}I-7S marker of purified human IgG coincided with the position of the purified Pseudomonas IgG antibody detected in gradient fractions by immunoprecipitation, regardless of its source. No smaller subunits of IgG were detected. To analyze further the purity and to investigate microheterogeneity of the IgG antibodies, analytical isoelectric focusing was performed in thin-layer 5% polyacrylamide gels. After staining the gels with 0.2% Coomassie Brilliant Blue, all specimens produced four distinct protein bands. The isoelectric points of these bands were equivalent from sample to sample and corresponded to the four control gamma subtypes of IgG. The microheterogeneity of the IgG opsonins with a

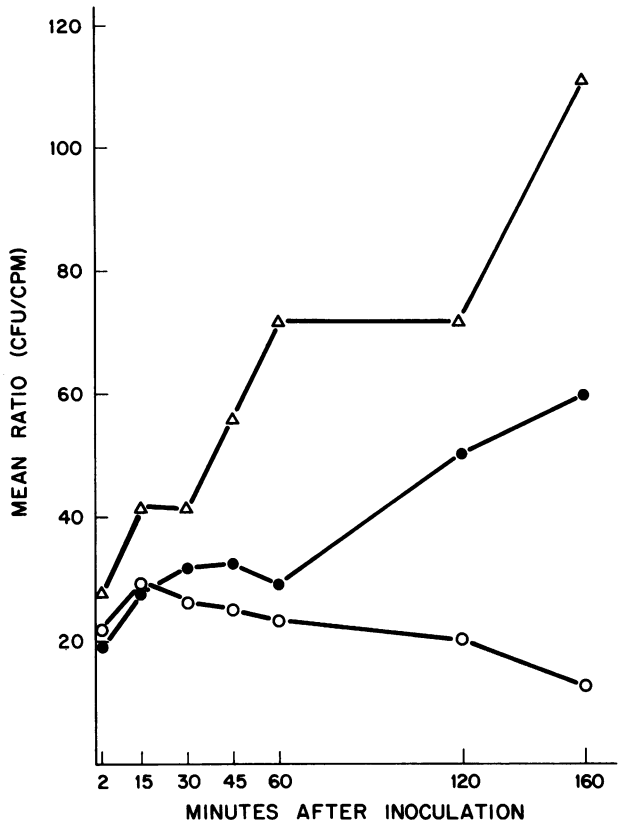

FIGURE 4 Efficacy of IgG opsonins (CF [△], HIS [○]) to sustain the combined effect of phagocytic uptake and intracellular killing. Opsonin-mediated phagocytic and bactericidal curves presented in Fig. 2 are shown here as the ratio of viable bacteria cultured from the monolayer (CFU) compared with the total bacteria uptake (^{14}C cpm) at various intervals after cell culture inoculation. With HIS-derived IgG opsonins, intracellular bactericidal mechanisms match total uptake and over the duration of the assay killing of Pseudomonas exceeds uptake and the CFU/cpm ratio falls. In constrast, CF opsonins produce a ratio that is steadily increasing and is greater at every point than the HA negative "opsonins" (●) or HIS samples. Bacterial survival in these CF cell cultures far exceeds actual Pseudomonas uptake, suggesting that less phagocytic uptake and macrophage ingestion is occurring.

high affinity for Pseudomonas became apparent when the isoelectric spectra were detected by use of radiolabeled LPS antigen and autoradiography (Fig. 5). The CF spectra (positions 1, 3–5, and 7) differ from the COLD (positions 2 and 6) and HIS (position 8) control in both the number of bands detected by this method and their relative intensity of radioactivity. A third band with a higher isoelectric potential and greater affinity for the iodoinated LPS is present in CF specimens but not in the controls. This may represent an additional IgG subtype with lipopolysaccharide reactivity present in CF sera preparations but absent in HIS specimens. If confirmed with purified IgG subclasses and formal K_m determinations the improved bacterial coating demonstrated in Fig. 6 would be readily explained.

Functional segments of the Pseudomonas antibody molecules. The abnormality in the phagocytic function of Pseudomonas IgG antibodies isolated from CF sera may be explained by either a defect in the coating of the Pseudomonas organism by this IgG (opsonization), or an inability of the opsonin-bacterial complex to attach properly to the AM cell membrane. The bacterial coating property of an antibody is attributed to its $F(ab')_2$ portion whereas optimal engulfment of the opsonized bacterium requires binding to the AM surface receptor that is mediated by the Fc portion of the IgG molecule. Accordingly, we next examined the functional integrity of both the Fab and Fc segments.

Pseudomonas opsonization. As expected all CF and hyperimmune control antisera when incubated with Pseudomonas type 4 and counterstained with FITC-conjugated anti-IgG produced positive fluorescence of the bacteria. Both the IgG fraction from a healthy adult without Pseudomonas precipitins (Table I) and a 0.9% saline solution served as negative controls. Immunofluorescence was present and opsonization was equally effective, whether the antiserum was heat inactived before incubation with Pseudomonas or the antiserum was originally derived from fresh or frozen sera. CF and HIS derived antisera had no inhibitory effect (judged by agglutination) on the number of viable bacteria in the inocula. The survival of such immunoglobulin-coated bacteria is shown in Fig. 2 (upper panel).

The degree of opsonization produced was quantitated by a SPA assay using ^{125}I-labeled SPA. The bacterial "coating" activity of various antisera, when mixed with Pseudomonas type 4 organisms and detected by ^{125}I-SPA, are compared in Table IV. For every microgram of type 4 specific IgG antibody from CF specimens added to the assay mixture, more SPA bound than for each microgram of antisera derived from standard hyperimmune sera. Moreover, the superior affinity

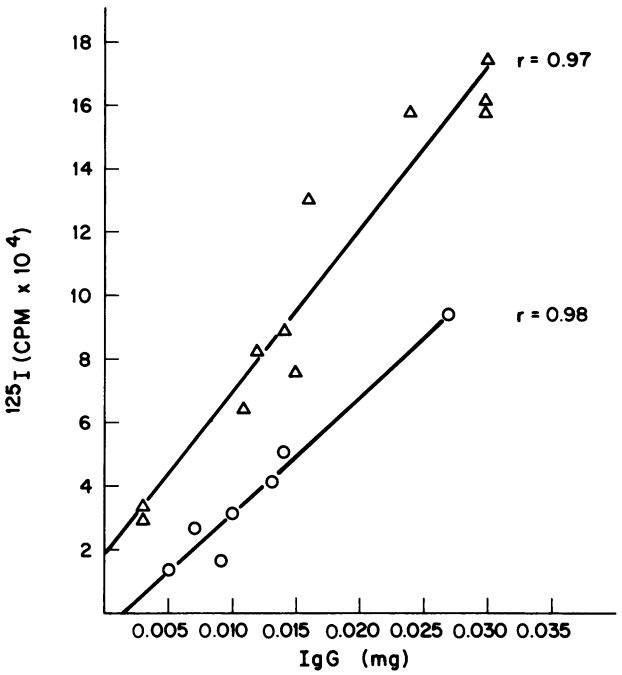

FIGURE 6 Effectiveness of bacterial "coating" with CF (△) and HIS (○) opsonins is shown to assess the $F(ab')_2$ function of the IgG molecules. This opsonic function was compared in a radioimmunoassay using Pseudomonas immunotype 4 as the solid phase and labeling the adherent CF and HIS opsonins with ^{125}I-SPA. The CF opsonins demonstrated a greater affinity for the bacteria in that every milligram of CF Pseudomonas specific IgG used in the assay (abscissa) resulted in a higher ^{125}I cpm (ordinate) than the comparably titered HIS derived antibodies. The *r* values represent the calculated Pearson's correlation coefficients.

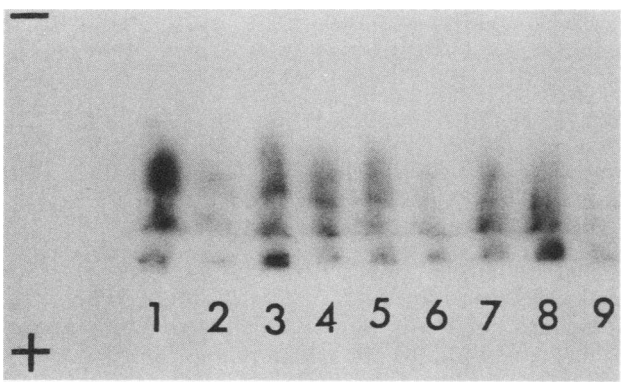

FIGURE 5 Isoelectric focusing and autoradiography. For electrofocusing of CF and control Pseudomonas IgG opsonins, samples were applied in equivalent protein concentrations to a 250-mm thin layer of 5% polyacrylamide gel with a carrier ampholyte gradient of pH 5–8. After treatment of the gel ^{125}I-LPS 4, the electrofocused antibodies were visualized by autoradiography. The five CF opsonins (positions 1, 3–5 and 7) demonstrate three bands with specificity for LPS 4, all of a greater intensity of radioactivity than COLD (positions 2 and 6) and negative control (position 9) opsonins. Only two bands are clearly delineated in the HIS (position 8)-derived Pseudomonas specific opsonins.

TABLE IV
Representative Experiment of Pseudomonas IgG Opsonin-Bacterial Adherence Using ^{125}I-SPA

Antibody source	IgG antibody used	^{125}I-SPA bound	Corrected	^{125}I-SPA bound/ μg IgG
	μg	cpm × 10^4	cpm × 10^{4*}	cpm × 10^5
Saline	0	0.74	0	0
Normal	52	4.31	3.57	6.87
HIS	9	4.02	3.27	36.38
CF_1	11	7.25	6.51	59.72
CF_2	15	8.36	7.62	51.47
CF_3	12	6.73	5.99	49.88
CF_4	30	16.09	15.35	51.15
COLD	21	11.69	10.95	52.13
CML	31	10.82	10.08	32.50

* Corrected for the nonspecific binding demonstrated in the saline control.

of CF opsonins could be demonstrated at various concentrations of IgG used in the assay (Fig. 6). This difference is reproducible, and shows a high level of statistical significance ($P < 0.01$) when ^{125}I-SPA cpm/mg IgG of HIS and CF are compared. Thus, coating or antibody attachment to the Pseudomonas strain was superior for CF IgG, and comparable for all of the other type-specific antibody preparations.

AM binding. The heat-killed Pseudomonas bacteria were directly labeled with FITC (F-Ps) without difficulty and retained the label when stored at −70°C for 14 d. The bacilli were brightly fluorescent and as a result binding of F-Ps to AM was easy to discern and unequivocal. Confirming the scanning electron microscopy data above, very few bacteria were visualized free in the AM monolayer after the routine washing procedure was performed just before the phagocytic-bactericidal assays. The mean percentage total AM per high power field that had F-Ps (≥1) associated with them at various points in the assay are diagrammed in Fig. 7. Although not systematically studied, few AM had >3 associated bacteria and neither the CF nor the HIS antisera resulted in >5 F-Ps/AM. Fluorescently labeled bacteria were taken up by the cell monolayers in a pattern similar to the ^{14}C-labeled bacteria (Fig. 1 and Fig. 2, lower panel). Despite adjustment to equivalent LPS HA titers, about twice as many F-Ps-AM complexes resulted when HIS-derived opsonins ($n = 3$) were used as compared to the CF IgG opsonins ($n = 6$). These differences were significant at 15- and 120-min intervals, ($P < 0.005$ for each). Despite high anti-Pseudomonas titers in the CF opsonic antibody samples, there were no statistically significant differences in the percentage of rosettes formed when

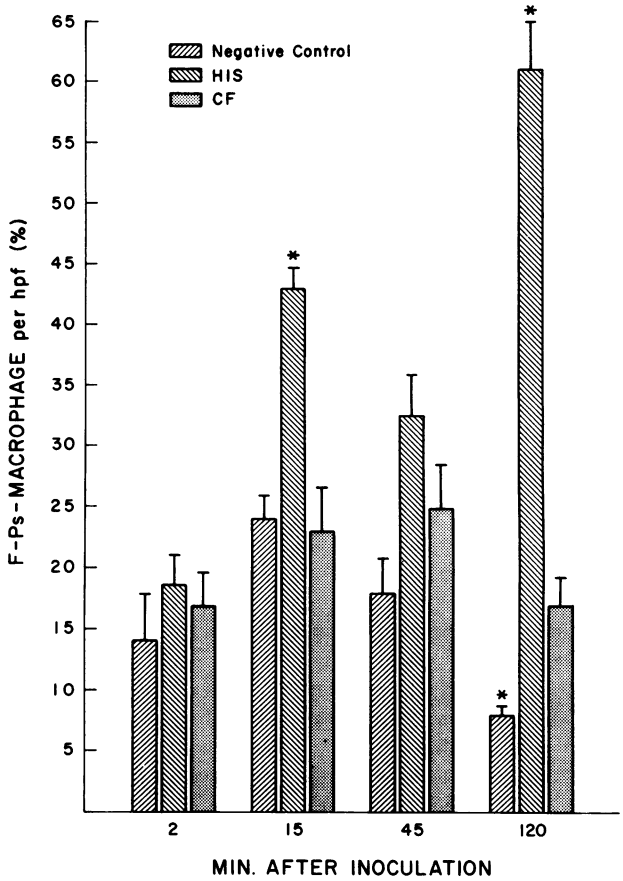

FIGURE 7 Binding of fluorescently labeled type 4 Pseudomonas (F-Ps) to AM membrane receptors are depicted. A representative experiment shows the effect of different IgG opsonins upon the percentage of F-Ps AM complexes formed (ordinate) at different periods of incubation (abscissa). The mean percentage of human alveolar macrophages per high power field (×630) binding labeled Pseudomonas coated with CF ($n = 6$), HIS ($n = 3$) equititered IgG opsonins, and negative control ($n = 3$) are compared. SEM is shown for each opsonin, and statistically significant differences ($P < 0.01$) when compared to CF IgG antibodies are designated (*).

compared to the GVBS buffer controls ($n = 3$) until 120 min. Whereas antibody coating (Table IV) had been found to be greater for the CF-derived IgG opsonins when compared to other immune opsonins, antibody attachment to the AM cell surface appeared less avid for the CF specimens.

DISCUSSION

The unusual predilection of CF patients to respiratory tract colonization with *P. aeruginosa* remains poorly understood. Because of reports concluding that whole serum from homozygous CF patients was deficient or inhibitory as a source of opsonins for phagocytosis and killing of Pseudomonas (6–9), we wished to pre-

pare IgG molecules with specific Pseudomonas LPS immunotype antibody activity. This opsonic defect seemingly was restricted to Pseduomonas organisms and to AM, for other gram-negative bacteria and normal peripheral PMN were unaffected by the presence of CF whole serum. Our assumption that IgG opsonins are inhibitory was bolstered by reports that demonstrated, convincingly, that serum factors are necessary for optimal phagocytosis of *P. aeruginosa*, and that in the presence of excess amounts of antibody, complement has no apparent role in the phagocytosis of Pseudomonas by rabbit AM (15). Boyden et al. (16) have reported that IgG antibodies in covalescent sera augment phagocytosis even in the absence of heat-labile opsonins. Some clinical data suggest the importance of the complement system in defense against bacteria, but the role of complement in pulmonary defense against Pseudomonas has not been convincingly demonstrated experimentally (34). Many in vitro and in vivo models have been used to analyze the role of complement in bacterial defense, but seldom has Pseudomonas been included. The CF strain of mucoid Pseudomonas has not been studied. These investigations have not been performed using human AM for phagocytic cells nor have they been performed in the complex milieu of the CF respiratory tract (35). No consistent alteration in serum complement levels have been documented in CF subjects and the amount in CF respiratory secretions is unknown (36, 37). Furthermore, IgG is superior to any of the other immunoglobulins in its bacterial opsonizing potential (11–14). Our assumption is further supported by the knowledge that immunoglobulin receptors on human AM are of the Fcγ class (18).

We chose a phagocytic assay composed only of human components so that proteins and cells from different animal species were not crossed. Biggar et al. (6) employed rabbit AM to judge the adequacy of human serum opsonins, concluding that CF serum was deficient in a phagocytic factor, probably IgA. Boxerbaum and co-workers (7) used a similar in vitro phagocytic assay comprised of rabbit AM and CF whole serum supplemented with rabbit immune serum. Their conclusion that a labile inhibitor was present in CF serum has been modified by more recent publications from their laboratory which report that this CF phagocytic inhibitor is heat stable (8, 9). When rabbit immunoglobulins have been used to opsonize Pseudomonas, which were then assayed in both mouse and canine phagocytic systems, there was an apparent loss in the potency of the opsonins (38). This may indicate a species specificity or sensitivity for cells and proteins. Therefore, the use of rabbit AM to assay the adequacy of human opsonins may not be an optimal combination.

To isolate anti-Pseudomonas IgG opsonins from CF patients, we chose an affinity chromatography method to enrich the antibody activity against naturally occurring Pseduomonas antigens in these subjects. Use of an LPS/substituted gel permitted the extraction of specific antibody from sera, providing an antibody reagent that theoretically does not contain extraneous, nonreactive, or nonimmune IgG molecules. The major immunodeterminant of opsonization of nonmucoid strains appears to be the immunotype antigen, and the use of type 4 LPS antigen in these experiments was particularly important because it represents one of the most frequent Pseudomonas immunotypes to which CF patients are exposed (2, 19). This somatic antigen is contained in the mucoid slime variant of *P. aeruginosa* that colonizes the respiratory tract of the majority of CF subjects and causes most of their lung infections.

We have chosen phagocytic and bactericidal assays that are well accepted and have been used extensively by many investigators (39–42). This dual assay of phagocytosis and intracellular killing measures both of these processes at the same time; it is the sum or outcome of these combined effects that may determine the morbidity and mortality from infection in CF patients. With the use of scanning electron microscopy these assays were modified to permit detection of the attached, but not internalized, fraction of the monolayer associated bacteria. Our result at 120 min of 6.2% is very similar to the 10% figure of Verhoef (43) and the 4% figure of Leijh and co-workers (29). This percentage is further supported by our studies employing Pseudomonas with a direct fluorescent label that allowed determination of monolayer associated background bacteria.

The results of the phagocytic and intracellular bactericidal kinetics potentiated by the various IgG opsonins were interesting. All of the bacterial uptake curves, irrespective of the opsonin source, demonstrated a brisk linear uptake over the first 60 min and then a slowing. This "satiety" effect may reflect what is already known about macrophage metabolism and antimicrobial mechanisms (35). Even under optimal conditions in vitro, no more than 50 or 60% of AM ingest viable Pseudomonas organisms (12). For this functionally active subpopulation, phagocytosis is an energy-dependent process requiring reactants that need to be regenerated and hence could be a rate-limiting process. Additionally, macrophage membrane receptors for the crystallizable fragment of IgG (Fcγ) are important in particle attachment and ingestion. During the uptake of large numbers of particles, phagocytes internalize a substantial quantity of plasma membrane. Although these receptors can be stimulated to increase in number by chronic antigen administration (44), time is necessary for membrane renewal. Use of CF-derived IgG opsonins specific for Pseudomonas immunotype

4 resulted in both decreased phagocytic rates and absolute bacterial uptakes that were depressed when compared to HA-matched positive controls and to negative controls matched in protein concentrations. The fact that bacterial phagocytosis accomplished with normal saline, also used as a negative control, was greater than the nine high titered CF opsonins, permitted us to conclude that CF IgG opsonins acted in an inhibitory fashion.

Another functional defect of CF opsonins, uncovered in the intracellular bacterial survival curves, is less impressive than the inhibition of bacterial phagocytosis, but seems distinctive. Despite the fact that CF opsonins depressed bacterial uptake, once internalization of CF opsonized bacteria had occurred, intracellular killing was sluggish. Total numbers of viable bacteria cultured from AM monolayer lysates exposed to the CF IgG antibodies were consistently higher than either positive or negative controls. The sum of these two effects, diminished uptake and less bacterial killing, was very significant (Fig. 4). The demonstration that intracellular bactericidal mechanisms are influenced by characteristics of the immunoglobulin serving as the opsonin has been reported recently by others (29) and is supported by current concepts of degranulation and secondary phagosome function. In contrast, the HIS-derived opsonins with equivalent type 4 HA titers facilitated a brisk intracellular bactericidal process. Once the HIS-opsonized bacteria had been internalized, the AM monolayers were able to attain an apparent equilibrium, allowing intracellular killing to keep pace with further ingestion of bacteria.

Two possible explanations for the diminished activity noted with the CF IgG opsonins encompass both functional ends of the molecule: (*a*) that the $F(ab')_2$ portion of the molecule has a low avidity for binding to Pseudomonas organisms, or (*b*) that the Fc portion attaches poorly to AM membrane surface receptors. Our radioimmunoassay using ^{125}I-labeled SPA as the marker and Pseudomonas type 4 as the solid phase demonstrated somewhat superior coating of the bacteria by the CF-derived molecules. However, when the opsonized bacteria were observed to interact with AM with the assistance of a direct fluorescent label, fewer bacteria were attached to AM membranes. This suggests that a defect in the Fcγ portion of the CF Pseudomonas-specific IgG is interfering with phagocytic function by preventing proper receptor attachment and subsequent triggering of internalization. IgG heavy chain subtypes 2 and 4 are not known to bind to mononuclear cells (45). Therefore, if the CF IgG opsonins were homogeneous, and comprised chiefly of one of these subtypes, no AM receptor binding would be expected. However, our immunochemical analysis of IgG subtypes revealed that the CF opsonin population is heterogenous and contains all four subtypes. An alteration, it should be noted, in the normal ratio of IgG_1 to IgG_2 to IgG_3 etc. could alter both the amount of SPA binding to total IgG and macrophage surface receptor attachment to such a degree that overall phagocytosis would be affected. Accordingly, we are developing an enzyme-linked immunoadsorbent assay to permit quantitation of IgG heavy chain subtypes.

These results may help to explain several clinical observations about CF. Although the exact role of bacteria in the initial pathogenesis of the CF pulmonary lesion has not been defined, it is clearly recognized that Pseudomonas infection is a major participant in the eventual pathologic lesion and hastens irreversible changes. In fact, evidence is accumulating that the pulmonary pathology is initiated by infection (46, 47). Pseudomonas colonization and the CF patient's clinical state are intimately related. Many clinicians have reported a direct correlation between the incidence of colonization and age, clinical score, extent of pulmonary disease, severity of radiographic changes, and level of serum immunoglobulins. There is a direct correlation between the presence, number, and titers of Pseudomonas precipitins and the severity of CF pulmonary disease. In spite of a pronounced humoral immune response, reflected by high levels of antibodies against Pseudomonas in serum and in sputum (48), these do not effectively promote elimination of the bacteria from the CF lung. Enhancement of these antibody titers by vaccination against Pseudomonas (19) does not alter the clinical course. It may be that in vivo these CF agglutinins inhibit macrophage phagocytosis, allowing Pseudomonas to persist in the respiratory tract and contribute to the well-characterized pathogenetic sequence of infection, obstruction, and atelectasis.

In addition immune complexes, both circulating and extravascular, have been described in CF by many groups (49). The possibility has been suggested that immune complexes might mediate pulmonary inflammation because the high antibody levels do not effectively clear the lungs of Pseudomonas and there is a strong correlation between this antibody response and the severity of the pulmonary disease. It may be that a defect in IgG Fc function with consequent decreased bacterial clearance could lead to prolonged circulation of such immune complexes in CF, thereby contributing to tissue deposition and damage.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of Mr. Alan S. Pooley in the performance of scanning electron microscopy. Suggestions from Drs. William Merrill, James E. Pennington, and Robert E. Wood were helpful in the course of this work. We also appreciate the help of Dr. Gerald J. Beck with statistical analyses and the expert secretarial assistance of Mrs. Mae P. Day.

This research was supported by grants from the Cystic Fibrosis Foundation and The National Heart, Lung, and Blood Institute, National Institutes of Health (22302L).

REFERENCES

1. Di Sant'Agnese, P. A., and P. B. Davis, 1976. Research in cystic fibrosis. *N. Engl. J, Med.* **295:** 481–485, 534–541, 597–602.
2. Zierdt, C. H., and R. L. Williams. 1975. Serotyping of *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis of the pancreas. *J. Clin. Microbiol.* **1:** 521–526.
3. Wallwork, J. C., P. Brenchley, J. McCarthy, J. D. Allan, D. Moss, A. M. Ward, A. Holzel, R. F. Williams, and H. McFarlane. 1974. Some aspects of immunity in patients with cystic fibrosis. *Clin. Exp. Immunol.* **18:** 303–320.
4. Halbert, S. P. 1967. Immunological aspects of cystic fibrosis. *Mod. Probl. Paediatr.* **10:** 144–157.
5. Wood, R. E., T. F. Boat, and C. F. Doershuk. 1976. Cystic fibrosis: state of the art. *Am. Rev. Respir. Dis.* **113:** 833–878.
6. Biggar, W. D., B. Holmes, and R. A. Good. 1971. Opsonic defect in patients with cystic fibrosis of the pancreas. *Proc. Natl. Acad. Sci. U. S. A.* **68:** 1716–1719.
7. Boxerbaum, B., H. Kagumba and L. W. Matthews. 1973. Selective inhibition of phagocytic activity of rabbit alveolar macrophages by cystic fibrosis serum. *Am. Rev. Respir. Dis.* **108:** 777–783.
8. Thomassen, M. J., B. Boxerbaum, C. Demko, P. Kuchenbrod, D. G. Dearborn, and R. E. Wood. 1979. Inhibitory effect of cystic fibrosis serum on Pseudomonas phagocytosis by rabbit and human alveolar macrophages. *Pediatr. Res.* **13:** 1085–1088.
9. Thomassen, M. J., C. A. Denko, R. E. Wood, B. Tandler, D. G. Dearborn, B. Boxerbaum and P. J. Kuchenbrod. 1980. Ultrastructure and function of alveolar macrophages from cystic fibrosis patients. *Pediatr. Res.* **14:** 715–721.
10. Høiby, H., and S. Olling. 1977. *Pseudomonas aeruginosa* infection in cystic fibrosis. Bactericidal effect of serum from normal individuals and patients with cystic fibrosis on *P. aeruginosa* strains from patients with cystic fibrosis or other diseases. *Acta Pathol. Microbiol. Scand. (C)* **85:** 107–114.
11. Young, L. S., and D. Armstrong. 1972. Human immunity to *Pseudomonas aeruginosa*. I. In vitro interaction of bacteria, polymorphonuclear leukocytes and serum factors. *J. Infect. Dis.* **126:** 257–276.
12. Reynolds, H. Y., and R. E. Thompson. 1973. Pulmonary host defenses II. Interaction of respiratory antibodies with *Pseudomonas aeruginosa* and alveolar macrophages. *J. Immunol.* **111:** 369–380.
13. 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 on the killing of bacteria. *Infect. Immun.* **4:** 462–467.
14. Reynolds, H. Y., J. A. Kazmierowski, and H. H. Newball. 1975. Specificity of opsonic antibodies to enhance phagocytosis of *Pseudomonas aeruginosa* by human alveolar macrophages. *J. Clin. Invest.* **56:** 376–385.
15. Murphy, S. A., R. K. Root, and A. D. Schreiber. 1979. The role of antibody and complement in phagocytosis by rabbit alveolar macrophages. *J. Infect. Dis.* **140:** 896–903.
16. Boyden, S. V., R. J. North, and S. M. Faulkner. 1976. Complement and activity of phagocytes, *In* Ciba Foundation on Complement, G. E. W. Wolstenholme and J. Knight, editors. Little, Brown and Company, Boston. 190–221.
17. Young, L. S., and D. Armstrong. 1972. Human immunity to *Pseudomonas aeruginosa* I. *In vitro* interaction of bacteria, polymorphonuclear leukocytes and serum factors. *J. Infect. Dis.* **126:** 257–276.
18. Reynolds, H. Y., J. P. Atkinson, H. H. Newball, and M. M. Frank. 1975. Receptors for immunoglobulin and complement on human alveolar macrophages. *J. Immunol.* **114:** 1813–1819.
19. Pennington, J. E., H. Y. Reynolds, R. E. Wood, R. A. Robinson, and A. S. Levine. 1975. Use of *Pseudomonas aeruginosa* vaccine in patients with acute leukemia and cystic fibrosis. *Am. J. Med.* **58:** 629–636.
20. Reynolds, H. Y., and J. S. Johnson. 1971. Structural units of canine serum and secretory immunoglobulin A. *Biochemistry.* **10:** 2821–2827.
21. Hanessian, S., W. Regan, D. Watson, and T. H. Haskell. 1971. Isolation and characterization of antigenic components of a new heptavalent pseudomonas vaccine. *Nature N. Biol.* **229:** 209–210.
22. Fraker, P. J. and J. C. Speck. 1978. Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a, 6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* **80:** 849–857.
23. Fick, R. B., G. P. Naegel, and H. Y. Reynolds. 1980. Use of *Pseudomonas aeruginosa* lipopolysaccharide and immunoadsorbents to prepare high potency, monospecific antibodies. *J. Immunol. Methods.* **38:** 103–116.
24. Reynolds, H. Y., and H. H. Newball. 1974. Analysis of proteins and respiratory cells obtained from human lungs by bronchial lavage. *J. Lab. Clin. Med.* **84:** 559–573.
25. Merrill, W. W., G. P. Naegel, R. A. Matthay, and H. Y. Reynolds. 1980. Alveolar macrophage-derived chemotactic factor: kinetics of in vitro production and partial characterization. *J. Clin. Invest.* **65:** 268–276.
26. Thomas, V., A. Shelokov, and M. Forland. 1974. Antibody-coated bacteria in the urine and the site of urinary-tract infection. *N. Engl. J. Med.* **290:** 588–590.
27. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I *BSA and antibody. *J. Infect. Dis.* **103:** 239–262.
28. Hinman, L. M., C. A. Stevens, R. A. Matthay, and J. B. L. Gee. 1980. Elastase and lysozyme activities in human alveolar macrophages. *Am. Rev. Respir. Dis.* **1212:** 263–271.
29. Leijh, P. C. J., M. T. Van Der Barselaar, T. L. Van Zwet, M. R. Daha, R. Van Furth. 1979. Requirements of extracellular complement and immunoglobulin for intracellular killing of micro-organisms by human monocytes. *J. Clin. Invest.* **63:** 772–784.
30. Gelfand, J. A., A. S. Fauci, I. Green, and M. M. Frank. 1976. A simple method for the determination of complement receptor-bearing mononuclear cells. *J. Immunol.* **116:** 595–599.
31. Phillips, M., and D. W. Dresser. 1973. Antibody isoelectric spectra visualized by antigen-coated erythrocytes. *Eur. J. Immunol.* **3:** 738–740.
32. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46:** 83–88.
33. Brown, B. M., and M. Hollander. 1977. Statistics: A Biomedical Introduction. John Wiley & Sons, Inc., New York.
34. Gross, G. N., S. R. Rehm, and A. K. Pierce. 1978. The effect of complement depletion on lung clearance of bacteria. *J. Clin. Invest.* **62:** 373–378.
35. Oren, R. A., E. Farnham, K. Sceito, E. Milofsky, and M.

Karnovsky. 1963. Metabolic patterns in three types of phagocytizing cells. *J. Cell. Biol.* **17**: 486–561.
36. Götz, M., and G. Lubec. 1978. Complement in cystic fibrosis. *Eur. J. Pediatr.* **127**: 133–139.
37. Conover, J. H., E. J. Conad, and K. Hirschhorn. 1973. Complement components in cystic fibrosis. *Lancet.* **II**: 1501.
38. Reynolds, H. Y., and R. E. Thompson. 1972. Canine respiratory macrophages. Diverse functions plus studies of bacterial phagocytosis and killing with serum and bronchial secretion immunoglobulins. *Fed. Proc.* **31**: 936a.
39. Cohen, A. B., and M. J. Cline, 1971. The human alveolar macrophage: isolation, cultivation in vitro, and studies of morphologic and functional characteristics. *J. Clin. Invest.* **50**: 1390–1398.
40. Rhodes, J. 1975. Macrophage heterogeneity in receptor activity: The activation of macrophage Fc receptor function in vivo and in vitro. *J. Immunol.* **114**: 976–981.
41. Rabinovitch, M. 1967. Studies on the immunoglobulins which stimulate the ingestion of glutaraldehyde-treated red cells attached to macrophages. *J. Immunol.* **99**: 1115–1120.
42. Czop, J. K., D. T. Fearon, and K. F. Austen. 1978. Opsonin-independent phagocytosis of activators of the alternative complement pathway by human monocytes. *J. Immunol.* **120**: 1132–1138.
43. Verhoef, J., P. K. Peterson, and P. G. Quie. 1977. Kinetics of staphylococcal opsonization, attachment, ingestion and killing by human polymorphonuclear leukocytes. A quantitative assay using [^{3}H]thymidine labeled bacteria. *J. Immunol. Methods* **14**: 303–311.
44. Arend, W. P., and M. Mannik. 1973. The macrophage receptor for IgG: number and affinity of binding sites. *J. Immunol.* **110**: 1455–1463.
45. Hay, F. C., G. Torrigiani, and I. M. Roitt. 1972. The binding of human IgG subclasses to human monocytes. *Eur. J. Immunol.* **2**: 257–261.
46. Lamb, D., and L. Reid. 1972. The tracheobronchial submucosal glands in cystic fibrosis: A qualitative and quantitative histochemical study. *Br. J. Dis. Chest.* **66**: 239–247.
47. Reid, L. 1980. Cardiopulmonary Pathology. *In* Perspectives in Cystic Fibrosis: Proceedings of the Eighth International Congress on Cystic Fibrosis. J. M. Sturgess, editor. The Imperial Press, Ltd., Ontario, Canada. 198–215.
48. Fick, R. B., and H. Y. Reynolds. 1980. Immunoprophylaxis and immune responses in cystic fibrosis. *In* Perspectives in Cystic Fibrosis: Proceedings of the Eighth International Congress on Cystic Fibrosis. J. M. Sturgess, editor. The Imperial Press, Ltd., Ontario, Canada. 335–345.
49. Moss, R. B., and N. J. Lewiston. 1980. Immune complexes and humoral response to *Pseudomonas aeruginosa* in cystic fibrosis. *Am. Rev. Respir. Dis.* **121**: 23–29.