Specificity of Opsonic Antibodies to Enhance Phagocytosis of *Pseudomonas aeruginosa* by Human Alveolar Macrophages

HERBERT Y. REYNOLDS, JOHN A. KAZMIEROWSKI, and HAROLD H. NEWBALL

From the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, and Pulmonary Branch, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT These studies compared the ability of specific secretory IgA (sIgA) and IgG antibodies to promote phagocytosis of viable *Pseudomonas aeruginosa* by human alveolar macrophages. Macrophages were obtained by lung lavage of normal adult smoker and nonsmoker volunteers and were maintained as in vitro cell monolayers. Both immune sIgA and IgG agglutinating antibodies were demonstrated to coat and opsonize viable bacteria, whereas similar nonimmune immunoglobulin preparations did not. When alveolar macrophages were challenged with viable opsonized ^{14}C-labeled Pseudomonas, IgG-reacted bacteria were ingested better and killed more readily than sIgA-opsonized organisms. Phagocytic responses were not significantly different between macrophages obtained from smokers and nonsmokers. Although sIgA and IgG antibodies can be found in respiratory secretions and both are undoubtedly important in pulmonary host defense, IgG opsonic antibody was superior in enhancing the uptake of *Pseudomonas* by in vitro-cultured alveolar macrophages. It may be the more important respiratory antibody for certain bacterial infections.

INTRODUCTION

A complex host defense system protects the lower respiratory tract from foreign particles inhaled with ambient air. Fortunately, various anatomic barriers in the upper airway and intricate branching of the tracheobronchial tree mechanically exclude particles larger than 3 μm in diameter from the respiratory bronchioles and alveoli (1, 2). However, smaller particles (0.5–3 μm), which may include infectious agents such as bacteria, can be deposited directly in the alveoli (1). This initiates a complicated, but still poorly understood, interaction between these potentially infectious particles, the protein-lipid-enzyme components of the alveolar surface, and the resident alveolar macrophages. Ideally, phagocytosis of the foreign particles by macrophages begins the clearance process.

In the present studies, two questions about the confrontation between bacteria and macrophages have been asked: (*a*) which opsonic antibody best facilitates bacterial phagocytosis, and (*b*) does the environmental background of the macrophage (i.e. whether the cells were obtained from cigarette smokers or nonsmokers) affect phagocytosis or killing of the bacteria? We have chosen *Pseudomonas aeruginosa* as the microorganism for study because it is a frequent cause of nosocomial pneumonia in patients with cardiorespiratory diseases (3–5) or with altered immunity (6).

METHODS

Normal subjects. Normal adult volunteers, hospitalized to participate in clinical projects at the National Institutes of Health, underwent limited bronchoscopy for selective lavage of the lingula lobe as described previously (7). Both cigarette smokers and nonsmokers were used. Informed consent was obtained. Each subject was premedicated with intramuscular atropine (0.5 mg) and diazepam (10 mg); local anesthesia of the respiratory tract was obtained with topical 2% lidocaine. Bronchoscopies were performed transnasally with a fiberoptic bronchoscope (model BF-T 5B2, Olympus Corporation of America, New Hyde Park, N. Y.). The bronchoscope was positioned in the lingula lobe orifice and aliquots of 50 ml of sterile saline (0.9% sodium chloride, Abbott Laboratories, North Chicago, Ill.) were infused through the bronchoscope and aspirated into a sterile container. Three 50-ml saline washings were done.

All subjects were examined regularly during the 48-h interval after bronchoscopy. Inspiratory rales were usually detected for 2–6 h in the lung area lavaged. All volunteers tolerated bronchoscopy and lavage well and had no noticeable effects from the procedures.

Recovery of respiratory cells. The lavage fluid was immediately strained through several layers of very loose cotton gauge to remove mucus and then centrifuged at 500 *g*

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for 5 min at 25°C. After the supernatant lavage fluid was decanted, the cell pellet was resuspended in modified Hanks' balanced salt solution (HBSS)[1] (prepared without Ca^{++} and Mg^{++} ions or phenol red), washed, and pelleted twice by centrifugation, before a final cell suspension was made.

Cell viability was assessed by eosin Y dye exclusion (8). Cells were counted (Coulter model F_n, Coulter Electronics Inc., Hialeah, Fla.) and 500 cell differential counts were done on wet mounts and cytocentrifuged Wright-Giemsa preparations. To size the various respiratory cells in the unstained wet mount, the diameter of a cell was measured in two planes under oil immersion (1,000 ×) and the average diameter was expressed in micrometers. Macrophages were identified by size and morphology, by staining with neutral red (9), and by ingestion of polystyrene latex balls (Dow Chemical Co., Midland, Mich.; mean diameter 1.1 μm).

Establishment of short-term macrophage cell cultures. Respiratory cells were cultured on glass surfaces in tissue culture chambers (No. 4802 two-chamber units, Lab-Tek Products, Div. Miles Laboratories Inc., Naperville, Ill.) with McCoy's 5A medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 0.3% vol/vol L-glutamine, 10% heat-inactivated fetal calf serum, and antibiotics—gentamicin sulfate (5 μg/ml) and penicillin G (100 U/ml). About 1.5×10^6 viable macrophages were added to each chamber and allowed to adhere in an atmosphere of moist air and 5% CO_2 at 37°C. Monolayers were allowed to acclimate for 24 h; then monolayers were washed three times with HBSS, with added Ca^{++} and Mg^{++}, and reconstituted with 1.9 ml of HBSS/chamber. Adherent cell monolayers consisted of approximately 95% alveolar macrophages. To insure comparability of cell cultures, individual chambers were checked for uniformity of the cell monolayers by examination with an inverted microscope at 200 ×; selected chambers were assayed for protein by the Lowry, Rosebrough, Farr, and Randall method (10).

Preparation of respiratory secretory IgA antibody. To obtain secretory IgA antibody (sIgA), respiratory secretions were collected from a patient, A. W. O. (NIH #0967865) after natural *Pseudomonas* pulmonary infection. The patient had chronic asthma and two previous episodes of *Pseudomonas aeruginosa* pneumonia. His respiratory secretions were watery and nonpurulent (produced 50–100 ml/day), and did not contain detectable IgM immunoglobulin. High titers of agglutinative antibody activity against *Pseudomonas aeruginosa,* immunotypes 3 and 7, were present.[2] Expectorated secretions were collected by postural drainage and immediately frozen to −20°C without preservatives. A pool of 500 ml of secretions collected during a 7-day interval was used in these experiments. The methods used to purify sIgA from bronchial secretions have been detailed previously (7). In brief, respiratory secretions were emulsified and centrifuged to obtain clear supernatant fluid (200 ml), which was then dialyzed extensively against borate saline buffer, pH 8.0 (12). After further dialysis in 0.02 M Tris-HCl (2-aminohydroxymethyl-1,3-propanediol), pH 8.0, the secretions were chromatographed on an anion-exchange column of DE-52 diethylaminoethyl cellulose (Whatman pre-swollen microgranular DEAE, H. Reeve Angel & Co., Inc., Clifton, N. J.) and eluted with a continuous salt gradient (12). Immunoglobulin-rich material eluted at conductivity between 3 and 17 mmho and pH 8.1–8.7. Immunoglobulin-containing effluent was concentrated and then gel-filtered through Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) with borate-saline buffer. In the column effluent, hemagglutinative antibody activity was present in the fractions containing IgA, which eluted just after the void volume of the column at about 38% of gel bed volume. These IgA-fractions were concentrated and aliquots were purified by ultracentrifugation in 5-ml 10–40% linear sucrose gradients (13). An ^{125}I-labeled rabbit IgG (14) marker protein was included in each sucrose gradient.

IgA protein had reactivity for secretory piece determinants and a sedimentation coefficient of approximately 11S. Upon immunoelectrophoresis at a variety of protein concentrations, antisera to whole human serum and colostrum (7) detected a single precipitin arc. An extinction coefficient of 12.37 ($\Sigma_{1\,cm}^{280\,nm,\,1\%}$) was used (15). The final sIgA preparation of 3.2 mg/ml had a hemagglutinative titer of 1:8192 against *Pseudomonas* immunotype 3 and 1:2048 titer against immunotype 7.

To obtain nonimmune sIgA, respiratory secretions from a patient (L. C. P., NIH #0995848) with chronic bronchitis and bronchorrhea, who had no antibody titers to *Pseudomonas* antigens, were collected and fractionated as described above. sIgA preparations were stored at 4°C in borate-saline buffer.

IgG antibody isolation from serum. Respiratory secretions used to isolate sIgA antibody contained IgG antibody as well, which eluted from Sephadex G-200 at about 52% of gel bed volume. However, mixed with the IgG were small amounts of monomeric 7S IgA (without secretory component determinants), which could not be satisfactorily removed. Therefore, serum from patient A. W. O. was used as the source of immune IgG opsonin (7); similarly, serum from L. C. P. was used for control nonimmune IgG.

Serum from clotted whole blood was precipitated with 30% dry ammonium sulfate. The serum precipitate was redissolved in borate saline buffer and dialyzed extensively to remove residual ammonium sulfate before dialysis against 0.02 M Tris-HCl. The ammonium sulfate fraction was chromatographed on DEAE and eluted with a 0.4 M NaCl-Tris gradient. Low molarity protein, conductivity between 1.0 and 9.0 mmho, at pH 8.5 was pooled and concentrated. Further purification was done in sucrose density gradients by ultracentrifugation. An extinction coefficient of 14.3 ($\Sigma_{1\,cm}^{280\,nm,\,1\%}$) was used for IgG (16). A final preparation of immune IgG (6 mg/ml) had *Pseudomonas aeruginosa* lipopolysaccharide hemagglutinative titers of 1:256 against immunotype 3 and 7. The nonimmune IgG at a similar concentration had titers of $< 1:4$.

Antisera and immunologic methods. Rabbit antisera against human serum, colostrum, and immunoglobulin G and A were obtained from Behring Diagnostics, American Hoechst Corp., Somerville, N. J. Sheep antiserum to IgA was supplied by Meloy Laboratories Inc., Springfield, Va. Secretory piece determinants on sIgA were detected with an absorbed sheep antiserum prepared against purified colostral IgA (M33A), kindly provided by Dr. D. S. Rowe (7). Lyophilized fluorescein-conjugated rabbit antisera to human IgA and IgG, absorbed to be heavy-chain-specific, were obtained from Behring. These antisera were absorbed extensively with heat-killed Pseudomonas, type 3, before use.

[1] *Abbreviations used in this paper:* HBBS, Hanks' balanced salt solution; s, secretory.

[2] Hemagglutination titers were kindly done by Dr. H. B. Devlin, Research and Development Division, Parke, Davis & Co., Detroit, Mich., as described (11).

Immunoelectrophoresis and double-diffusion immunoprecipitation were done with standard methods, referred to previously (7).

Radioactive bacteria. *Pseudomonas aeruginosa,* immunotype 3 in the Fisher, Devlin, and Gnabasik typing scheme (17), was obtained as a lyophilized culture from Parke, Davis & Co. (lot 05074). *Pseudomonas aeruginosa* organisms were inoculated into 25 ml of trypticase soy broth containing 0.05 mCi L-[^{14}C]amino acid mixture (New England Nuclear, Boston, Mass.) and grown for 18–20 h at 37°C. The bacteria were sedimented by centrifugation at 3,000 *g* for 10 min and resuspended in 40 ml of 0.85% saline by vigorous mixing. Such washings were repeated four or five times until radioactivity in the supernatant fluid was minimal. After the final wash, about 90% of the radioactivity was associated with the bacteria. The bacteria were suspended to 10^8 organisms/ml by optical density at 620 nm; concentrations were confirmed by quantitative pour plate cultures. Bacteria remained in the lag phase of growth throughout each experiment.

Opsonization of bacteria. Washed suspensions of ^{14}C-labeled *Pseudomonas* organisms in saline (10^8/ml) were added to equal volumes of specific immunoglobulin preparations used as sources of opsonic antibody. The mixtures were incubated at 37°C for 15 min. Bacterial opsonization was always done in a slight excess of antibody protein, and incubation times were limited so that visible aggregation of bacteria would not occur. The opsonized bacteria were not washed to remove nonreactive protein because centrifugation often promoted macroscopic agglutination. Instead, the opsonized bacteria (0.1-ml inoculum) were added to an approximately 20-fold excess volume of HBSS in the cell culture to dilute unreactive protein to insignificant amounts.

To insure that bacterial opsonization or coating had occurred with immune IgG and sIgA, three methods were used to detect antibody associated with the *Pseudomonas* organisms. Viable bacteria (10^9/0.5 ml) were reacted for 15 min with the particular immunoglobulin preparation, either immune or nonimmune, then pelleted by centrifugation (3,000 *g* for 15 min), washed, and finally repelleted and inactivated with 10 μg/ml concentration of gentamicin. The first method was to disrupt the *Pseudomonas* pellet by sonication with a 3:1 volume of glass powder on ice at 80 W for 60 s (Sonifer Cell Disruptor, Model W185D, Ultrasonic Systems, Inc., Farmingdale, N. Y.), and then to examine the pellet for antibody with specific antiserum by double-diffusion immunoprecipitation. With the second method, *Pseudomonas* organisms were reacted with an equal volume of fluorescein-conjugated rabbit anti-human IgG or IgA antiserum (diluted 1:5 in saline) for 30 min at 37°C and then for 18 h at 4°C. After the bacteria were rewashed three times, they were examined for immunofluorescence at 500 X (Carl Zeiss, Oberkochen, Württenberg, West Germany, large fluorescent microscope, mercury arc lamp-HB0200, vertical illuminator with excitation filters of interference blue KP 490 and KP 500, dichoric reflector 500, and barrier filter LP 520). In the third method, antibody was absorbed to the inactivated *Pseudomonas* organisms and eluted from the bacteria with a method modified from that described by Eddie, Schulkind, and Robbins (18). The bacterial pellet was suspended in 1 ml of 0.1 M citrate buffer, pH 2.2, for 90 min at 37°C and then centrifuged, and the supernate was immediately neutralized with 2 M Tris, pH 10.5. The supernate was concentrated to about 0.1 ml volume with negative pressure dialysis and then analyzed with specific antisera by double-diffusion immunoprecipitation.

Bacterial uptake and killing assay (19). Opsonized radiolabeled *Pseudomonas* (0.1 ml) were added to the supernate (1.9 ml HBSS) of the macrophage monolayers and the chambers were reincubated at 37°C with intermittent shaking. The ratio of bacteria to cells was set at 10:1. At varying intervals duplicate chambers were selected, the supernates were decanted, and cell layers were washed repeatedly three to four times with 2-ml portions of HBSS. Then the cell layers were lysed with 2 ml of distilled water for 15 min and scraped with a rubber policeman. A sample (0.1 ml) was aspirated for bacterial culture and the remainder of the cell layer homogenates was transferred to glass counting vials and dried at 85°C. The dried cell homogenates were digested with 0.5 ml of 0.2 N NaOH for 2 h at 37°C, after which the mixture was neutralized with 0.2 ml of 3% acetic acid, and 10 ml of Aquasol (New England Nuclear) were added. Occasionally, about 0.5 ml of distilled water was added to the vials to improve solubility of the sample in the scintillation fluid. Vials were counted for ^{14}C activity in a liquid scintillation spectrometer (Packard Tricarb model 3375, Packard Instrument Co., Inc., Downers Grove, Ill.), and net counts expressed in counts per minute. Vials were counted for 10 min so that the standard error was less than 1%; counting efficiency was approximately 65%.

The cell homogenate sample for bacterial culture was serially diluted in 0.01% human albumin (Abbott Laboratories, Diagnostics Div., South Pasadena, Calif.) and distilled water and enumerated by quantitative pour plates with trypticase soy agar. The number of colony-forming bacteria was counted at 24 and 48 h; the definitive count was obtained from the pour plate having approximately 100 colonies.

Monolayers selected for phagocytic indexes were scanned with phase contrast microscopy before the cell chambers were air-dried and stained with Wright-Giemsa stain. 500 macrophages selected from at least five high-power microscopic fields (oil immersion, 1,000 ×) were counted for the presence or absence of intracellular bacteria. Those with ingested bacteria were divided further into those with one to four bacteria per cell and those with five or more per cell, and these were expressed as a percentage of the number of macrophages with intracellular organisms. The identity of stained dishes was unknown during counting, and dishes were chosen at random.

Thus, to summarize the design of the phagocytosis and bacterial assay: Cell monolayers were inoculated simultaneously with opsonized ^{14}C-labeled *Pseudomonas,* and during the next 60–120 min duplicate dishes from immune and control groups were selected at intervals and sampled as described. The efficiency of the macrophage monolayer to handle the bacterial challenge was evaluated by three parameters: (*a*) ^{14}C counts in the cell layer homogenate; (*b*) number of viable bacteria associated with the cell layer; and (*c*) visual estimate of phagocytosis by determining a phagocytic index from stained cell monolayers taken from each group at 30 and 60 min after inoculation. No antibiotics were used in the assay.

RESULTS

The respiratory cell recovery is contrasted for the groups of smokers and nonsmokers in Table I. The mean age of the subjects was 21±1.0 yr (range 19-23 yr). The degree of cigarette smoking for the smokers was about 1-3 pack-yr; no volunteers had evidence of

Table I

Cell Recovery and Cell Types in Bronchial Lavage Fluid from Smoker and Nonsmoker Subjects

Groups	Number respiratory cells	Viability	Macrophages	Lymphocytes
	$\times 10^6$	%	%	%
Smokers	39.0±5.3*	94.2±1.5	89.8±4.2	7.2±1.8
(n = 5)	(24.7–54.0)‡		(74–96)	(4–12)
Nonsmokers	13.6±2.3	93.0±2.3	78.4±3.1	18.0±2.2
(n = 6)	(6.0–21.1)		(72–89)	(12–25)

* Mean ± SEM.

‡ Range observed for each group.

pulmonary disease and all ventilatory function tests were normal.

The recovery of infused lavage fluid was about 70± 8.0% (range 50-93%) and was the same for both groups. About three times as many respiratory cells were obtained from smokers as from nonsmokers, and there were significant differences in the cellular composition of the lavage fluid. The nonsmokers had a greater percentage of lymphocytes in the cell differential and a correspondingly lower percentage of macrophages than the smokers (7). However, the size distribution of macrophages by average cell diameter in a wet cell mount did not differ between smokers and nonsmokers, with about 70% of the macrophages of intermediate size (13-19 μm diameter), about 20% of 10-12 μm in diameter and morphologically similar to peripheral blood monocytes, and about 5% as giant cells (> 20 μm diameter) with multiple nuclei. Polymorphonuclear cells were found in less than 3% and basophil-mast cells were observed very infrequently.

The number of alveolar macrophages obtained from an individual was only sufficient, usually, to allow one complete bacterial uptake study comparing two opsonin preparations. This limitation was particularly true for the nonsmokers, from whom fewer respiratory cells were recovered.

Evidence that immune IgG and sIgA opsonized (coated) Pseudomonas organisms. Washed, viable type 3 *Pseudomonas aeruginosa* were incubated with varying concentrations of immune and nonimmune IgG and sIgA. Control nonimmune immunoglobulin preparations could not be detected on bacterial surfaces by immunofluorescence nor eluted from these cells. Thus, nonimmune immunoglobulins did not stick nonspecifically to the *Pseudomonas* organisms and opsonization did not occur. In contrast, purified immune IgG and sIgA antibodies were adherent to bacteria, as demonstrated by immunofluoresence, and could be recovered from aggregated *Pseudomonas* organisms. The fluorescent assay was the most sensitive method used. Immune IgG, reacted with 10^9 bacteria in a concentration range of 0.2-6.0 mg/ml, was detected on bacterial surfaces at 0.4 mg/ml dilution. sIgA antibody at 0.8 mg/ml gave positive results with a comparable number of bacteria. For the phagocytic experiments, however, greater concentrations of antibody and fewer bacteria (10^8) were used to insure moderate-to-heavy coating of bacteria, rather than minimal opsonization just described.

Background counts. In a protein-free medium (HBSS), a large percentage (50–70%) of glass-adherent alveolar macrophages ingested inert polystyrene balls as a consequence of a process termed nonimmune endocytosis (20). In contrast, only about 5% of macrophages would phagocytose washed and viable nonopsonized *Pseudomonas* organisms in a HBSS medium that did not contain additional protein. Therefore, the background uptake of viable bacteria by alveolar macrophages was small in the absence of specific antibodies or opsonins, and the enhancing effect of any specific opsonin was usually striking. Another factor contributing to base-line bacterial counts in this macrophage assay system was the nonspecific sticking of bacteria to the glass surface. If washed ^{14}C-labeled *Pseudomonas* were added to a culture chamber in 1.9 ml of HBSS and in the absence of alveolar macrophages, a small but linear deposition of bacteria on the glass surface occurred. 60 min after chamber inoculation, about 800-1,000 ^{14}C cpm could be recovered after the glass surface was washed several times with medium and scraped with a rubber policeman. Bacteria opsonized with immune IgG or sIgA were no more adherent to the glass than nonopsonized ones.

Comparison of immune IgG opsonin and control IgG. The opsonic effect of immune IgG and nonimmune control IgG of promoting *Pseudomonas* uptake by macrophages obtained from nonsmokers and smokers was compared. Three similar experiments for each category of nonsmoker and smoker were performed; representative results are depicted in Figs. 1 and 2. The hemagglutinative titer of immune IgG at the concentration used was

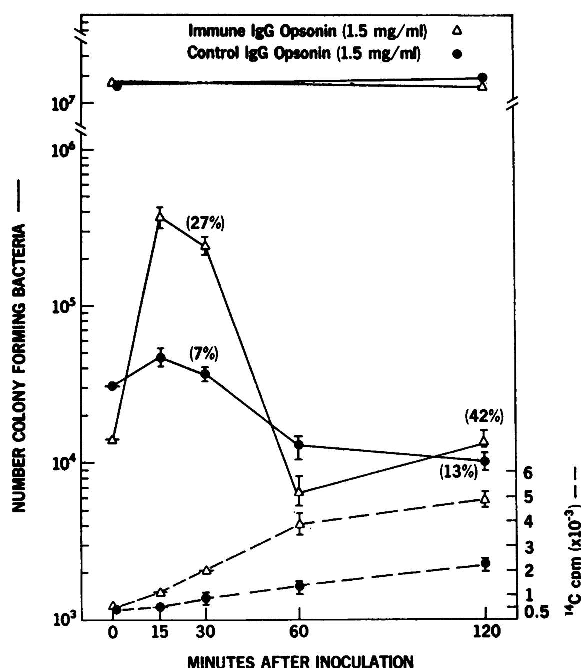

FIGURE 1 1.5 mg/ml immune (△) and 1.5 mg/ml nonimmune (●) IgG opsonins, isolated from serum, are compared. The *Pseudomonas*, immunotype 3, inocula incubated with the opsonin preparations are shown above the break in the long scale for viable bacteria. An inoculum of 2×10^7/0.1 ml of opsonized *Pseudomonas* was added to each macrophage chamber. Viable bacteria (——) and ^{14}C counts (– – –) for total bacteria in the cell homogenates are shown for duplicate monolayers sampled at intervals during a 2-h observation period. Phagocytic indexes were calculated at 30 and 120 min after inoculation. Macrophages were obtained from a nonsmoker.

1:32; the titer of the control IgG was $< 1:2$. In Fig. 1, the activity of macrophages from a nonsmoking subject is shown. The format of this particular experiment differs from others in that observations were continued for 2 h after bacterial inoculation of the cell monolayers. The viability of opsonized bacterial inocula, shown above the break in log scale, left ordinate, were unchanged during the assay and indicated that the *Pseudomonas* organisms remained in lag growth phase. The uptake of bacteria by both groups of cell monolayers was nearly linear during the entire 2-h observation, but the most striking increase occurred by 60 min for the immune IgG-treated organisms. ^{14}C bacterial counts were approximately two times greater at 60 min for the macrophages exposed to the immune IgG-treated organisms and a similar relationship was maintained at completion of the study.

The higher ^{14}C bacterial counts were corroborated by higher phagocytic indexes found at 30 min and 120 min for the immune IgG-challenged cell monolayers. It might be emphasized that the phagocytic index was based on the percentage of macrophages that appeared to have intracellularly located bacteria, as determined from Wright-Giemsa cell stains. The dynamics of the phagocytic process were better appreciated in a preliminary study of the cell monolayers by phase contrast microscopy. Under phase, the continuum between bacterial attachment to the macrophage cell surface and subsequent bacterial internalization could be followed. Not only did the immune IgG-*Pseudomonas*-exposed cell monolayers have more intracellular bacteria, but they had many more surface-adherent bacteria than the control IgG macrophages. Thus, many more bacteria were associated with the macrophages in the immune IgG opsonin group than in the control group. This increase was also reflected in the initially high bacterial colony counts obtained for the immune IgG exposed monolayers at the 15- and 30-min sampling times. Thereafter by 60 min, the viable bacterial count had decreased by more than a factor of 10. Macrophages were apparently killing *Pseudomonas* organisms more rapidly than they were being accumulated by the cell monolayers. To a much lesser degree the same results were obtained with the control IgG-*Pseudomonas*-exposed monolayers but in no instance did any of the parameters of bacterial uptake or killing approach those obtained in the immune IgG-exposed monolayers. In most respects, the control IgG-treated *Pseudomonas* produced base-line values similar to those of nonspecific bacterial deposition (about 1,500 ^{14}C counts at 60 min) and small phagocytic indexes.

For contrast, the response of a smoker's alveolar macrophages is shown in Fig. 2, with the same IgG preparations at the same concentrations described in Fig. 1. Observations were continued for 60 min after bacterial inoculation in this experiment. Total bacterial uptake was appreciably greater by monolayers exposed to the immune IgG-opsonized *Pseudomonas* and at 60 min the ^{14}C counts were about four times greater than the controls. Likewise, higher phagocytic indexes were found for the macrophages infected with the immune IgG-treated bacteria. Approximately 10% of the macrophages that had ingested immune IgG-coated bacteria by 60 min contained five or more organisms per macrophage; whereas, only 1% of the cells exposed to the nonimmune opsonized bacteria had as many intracellular bacteria. Phagocytic indexes for these IgG control monolayers of 5 and 13% at 30 and 60 min were approximately background values. The viable bacterial counts (left ordinate) in the immune IgG-challenged monolayers increased exponentially during the first 45 min of the assay, but at 60 min viable bacteria had begun to decrease, despite continued accumulation of ^{14}C bacterial counts. The pattern of these assays has shown that 30-45 min are required before any bacterial killing

is evident; thereafter, the viable bacterial counts may decrease significantly, as shown in Fig. 1.

A comparison of the macrophage responses obtained with nonsmoker (Fig. 1) and smoker (Fig. 2) cells shows many similar results. The uptake of immune IgG opsonized ^{14}C bacteria was comparable for both groups of macrophages, particularly during the first 60 min of uptake when the regression slopes of the uptake curves were almost the same (0.028±0.006 SE vs. 0.023±0.006). Phagocytic indexes were quite similar Because nonimmune IgG failed to opsonize the *Pseudomonase* organisms adequately, bacterial uptake (^{14}C) and phagocytic indexes were not above background values. Although the viable bacterial counts of immune IgG-reacted *Pseudomonas* at 60 min were lower for nonsmoker macrophages (Fig. 1) than for smokers' macrophages (Fig. 2), this apparently enhanced bacterial killing was not substantiated in other experiments. In essence no striking differences were found to distinguish the responses of smoker and nonsmoker alveolar macrophages.

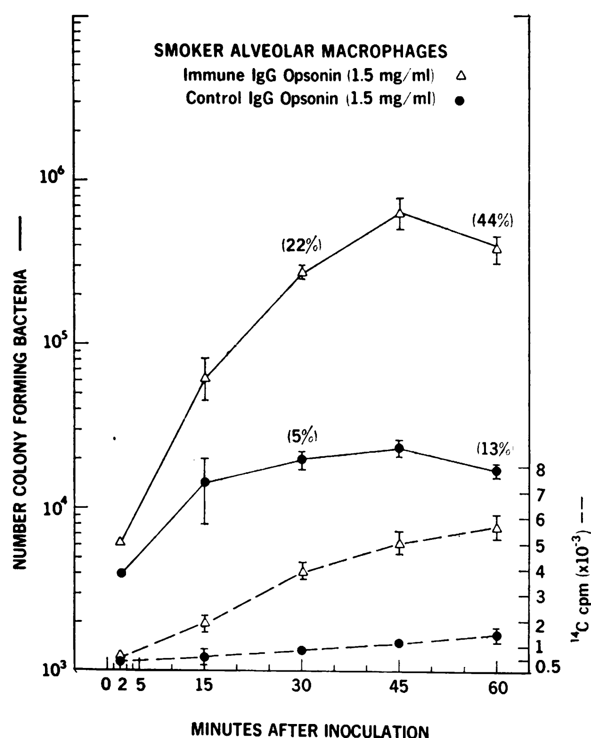

FIGURE 2 The response of alveolar macrophages from a smoker is shown with immune and control IgG-reacted type 3, ^{14}C-labeled *Pseudomonas* (2×10^7/0.1 ml inoculum/chamber). Duplicate monolayers from each group were sampled at intervals after inoculation for viable colony-forming bacteria (left ordinate) and total [^{14}C]bacteria (right ordinate). Phagocytic indexes are given in parentheses.

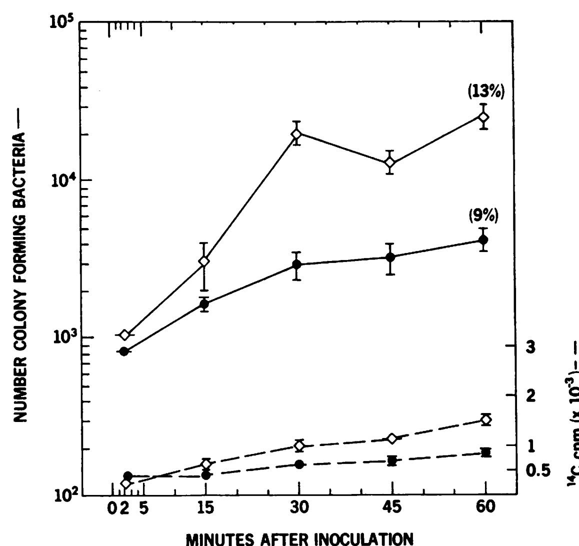

FIGURE 3 The ability of 0.8 mg/ml immune (◇) and 0.7 mg/ml nonimmune (●) sIgA to promote alveolar macrophage uptake of *Pseudomonas aeruginosa,* immunotype 3, is shown. Viable [^{14}C]bacteria were added (1.7×10^7/0.1 ml) to the supernatant fluid of cell monolayers; duplicate chambers were assayed at intervals after inoculation. Solid lines denote the number of colony-forming bacteria cultured from the cell homogenates; dashed lines show total [^{14}C]bacteria in the cell homogenates as radioactivity on the right ordinate. The percentage of macrophages in the monolayer having phagocytized bacteria at 60 min is given in the parentheses (phagocytic index). Macrophage viability at the end of the 60-min observation period was 90%. Cells were lavaged from a smoker.

Studies with sIgA. The effects of immune and nonimmune secretory IgA antibody to enhance bacterial uptake were evaluated with macrophages obtained from two smokers. One such comparison is shown in Fig. 3. The IgA preparations were compared at approximately the same protein concentrations. Each monolayer was inoculated with a 10:1 ratio of opsonized *Pseudomonas* to macrophages, and duplicate chambers were sampled from each group at 2 min after infection and at 15-min intervals for 1 h. Bacteria, as shown by ^{14}C counts, were taken up by the cell monolayers in roughly linear fashion and about a thousand ^{14}C counts were accumulated by both groups of cell monolayers at 60 min. Although more viable *Pseudomonas* were cultured from the immune sIgA-exposed monolayers, the phagocytic indexes were comparable for each group. In essence, the immune sIgA only gave a modest increase in macrophage uptake, compared with the control. It should be emphasized that *Pseudomonas* reacted with sIgA antibody did have demonstrable antibody identified and eluted from the bacteria, so that opsonization in fact occurred. In contrast, nonimmune sIgA did not coat the *Pseudomonas.*

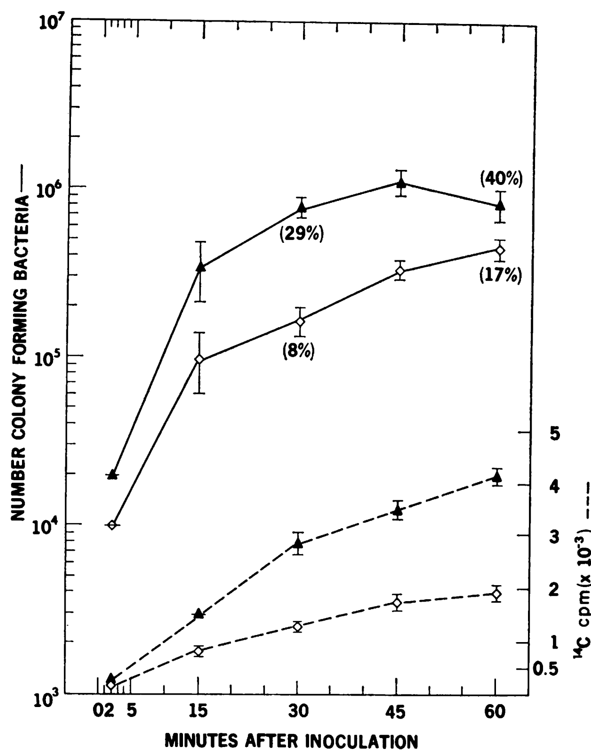

FIGURE 4 1.4 mg/ml immune sIgA (◇), isolated from respiratory secretions, and 1.2 mg/ml immune IgG (▲), isolated from serum, each had hemagglutinative antibody activity for *Pseudomonas,* immunotype 3. The two antibody preparations were compared at approximately equal protein concentrations for opsonic activity. An inoculum of 1.5 × 10^7/0.1 ml of opsonized viable ^{14}C-labeled *Pseudomonas* was added to each monolayer. Duplicate monolayers from each group were selected at intervals during the next 60 min for culture of viable bacteria (——) and for total ^{14}C bacterial counts in the cell homogenates (- - -). Respective phagocytic indexes are shown in parentheses. Macrophages were lavaged from a smoker.

These results with immune and nonimmune sIgA are similar to the background values obtained with this experimental system. Nonspecific bacterial attachment to the glass surface of the macrophage chamber will result in comparable ^{14}C bacterial counts, despite repeated and vigorous washing of the macrophage monolayers to remove extraneous bacteria. Phagocytic indexes of about 5-8% can be calculated when washed nonopsonized, viable *Pseudomonas* are added.

A direct comparison of immune sIgA and immune IgA opsonins was made with macrophages obtained from three subjects: two smokers and a nonsmoker. In the representative experiment illustrated by Fig. 4, alveolar cells were obtained from a smoker. Both opsonins were used at approximately the same protein concentrations; IgA had an agglutinative titer of 1:1,600 and IgG a titer of 1:32 against immunotype 3 *Pseudomonas aeruginosa,* the same organism used in the other experiments. During the 60 min of this assay, the ^{14}C bacterial uptake was twice as high in the monolayers exposed to IgG-treated bacteria as in the sIgA-challenged group. This difference was reflected in the higher phagocytic indexes calculated at 30 and 60 min for the IgG group. Phase contrast microscopy revealed many more macrophage cell surface-associated bacteria in the IgG-exposed monolayers. From the stained monolayer preparations made at 60 min, 14% of the IgG-exposed macrophages with intracellular *Pseudomonas* (phagocytic index 40%) had ingested five or more bacteria per cell. In contrast, only 2% of the IgA challenge macrophages (phagocytic index 17%) contained five or more bacteria. The number of viable bacteria cultured from the cell homogenates was higher at each sampling interval for monolayers exposed to IgG-opsonized bacteria, consistent with the greater number of total ^{14}C bacteria found associated with this group. However, at 60 min the colony counts decreased for the IgG-challenged cell monolayers, suggesting that *Pseudomonas* killing was beginning; such a finding was not observed for the IgA-exposed cells. Therefore, it was clear that sIgA was less effective than IgG in promoting bacterial uptake under these experimental conditions.

Two additional points should be considered. First, sIgA has been examined in this macrophage assay system at various protein concentrations that ranged from 0.8 mg/ml (Fig. 3) to the maximum concentration of 3.2 mg/ml. Results with immune sIgA-opsonized bacteria were not improved with respect to control nonimmune sIgA or to immune IgG values. Second, when sIgA concentrations greater than 2.0 mg/ml (or agglutinative titer 1:2,048) were used in opsonization studies, viability of the *Pseudomonas* inoculum (10^7/0.1 ml) decreased about 50% during the 60-min interval of the experiment, because of presumed bacterial clumping. If clumps of bacteria were added to the cell culture supernate, macrophage phagocytosis was generally less.

DISCUSSION

An important aspect of this study was the attempt to identify the immunoglobulin class that provides the most efficient opsonic antibody for certain bacterial infections of the lung. This finding could influence the route of immunization for bacterial vaccination or the products used for passive antibody administration. Although interpretation of the results must be limited because of the in vitro design of the experiments and the use of a single bacterial species, efficacy of IgG antibody to enhance *Pseudomonas* uptake was apparent.

The specific immunologic recognition of IgG-coated viable bacteria must be distinguished from the nonimmunologic phagocytosis (20) of inanimate particles

such as polystyrene balls. Less than 10% of the alveolar macrophages in a monolayer would ingest washed viable *Pseudomonas* organisms in a protein-free medium; whereas more than 50% ingested polystyrene balls under similar conditions. With appropriate opsonization with immune IgG, 30-40% of the macrophages would ingest viable *Pseudomonas* within 30-60 min after bacterial challenge. In contrast, nonimmune or control IgG does not effectively interact or coat the bacteria, and subsequent phagocytosis is no better than that obtained with unsensitized *Pseudomonas* in a protein-free medium (5-13%). Alveolar macrophages have cell surface receptors for IgG (21, 22) and are able to phagocytose IgG antibody-coated erythrocytes and bacteria. Thus, the results obtained with IgG-opsonized *Pseudomonas* were largely predictable from knowledge of the specificity of the antibody and the appropriate receptor of the alveolar macrophages.

sIgA-opsonized *Pseudomonas* behaved in vitro as if alveolar macrophages did not have an attachment site or specific receptor for this immunoglobulin. Admittedly this conclusion is inferred, because IgA receptors on alveolar macrophages have not been studied, to our knowledge. Alternatively, the fault could lie with the opsonization potential of the sIgA antibody. Recent studies (23) have found that 11S human sIgA antibody (anti-A isoagglutinins purified from colostrum) did not opsonize type A erythrocytes for phagocytosis by either monocytes or polymorphonuclear leukocytes. Poor sIgA coating of the *Pseudomonas* organisms was not considered a problem in our studies. After brief antibody reaction with the *Pseudomonas,* as done for 15 min in these studies, and washing the bacteria, sIgA could be still detected immunologically on the organisms. Thus, at best, after 60 min of opportunity, only 13-17% of monolayer macrophages ingested sIgA-opsonized *Pseudomonas* (Figs. 3 and 4). In addition, bacterial-macrophage surface attachment, as viewed with phase contrast microscopy, was much less evident than that seen with IgG-opsonized bacteria.

Macrophage interaction with IgM-opsonized *Pseudomonas* was not included in these studies because it has been amply documented (7, 24-26) that lower respiratory tract secretions of normal subjects do not contain detectable amounts of IgM. Furthermore, the unlikely importance of IgM opsonic antibody in the diseased lung is illustrated by the fact that human alveolar macrophages do not have a cell membrane receptor for IgM antibody and are, therefore, unable to ingest IgM-coated particles (21). In addition, polymorphonuclear leukocytes that readily enter infected pulmonary tissue do not have IgM receptors either (23, 27).

In a healthy human, the IgG present in pulmonary secretions may originate from two sites, local synthesis in the submucosa of the respiratory tract or the intravascular globulin pool. With immunofluorescent techniques, IgG-producing plasma cells have been identified in human respiratory tissues (28, 29) in numbers approximately equal to IgA cells. Moreover, cell cultures of bronchial wall and lung tissues are capable of synthesizing IgG and IgA proteins (30). In addition, intravascular IgG apparently diffuses into respiratory secretions. If this passive diffusion in humans is analogous to that observed in animals, about 1% of a parenteral dose of homologous ^{125}I-labeled IgG can be recovered from rabbit lung washings (31). In normal dogs, about 0.1% of the parenteral dose of ^{125}I-labeled IgG is recovered in serial bronchial washings (J. Kazmierowski, unpublished observations). These experimental diffusion studies of IgG only provide some rationale for the well-documented clinical use of periodic gammaglobulin injections in patients with hypo- or agammaglobulinemia (32). These passive immunizations seemingly decrease the incidence of bacterial pulmonary infections in these patients. Although commercial gamma globulin preparations consist primarily of IgG globulin (33), IgG antibody may be the most important component. Its superior opsonic activity and specific receptor attachment to lung macrophages could be the explanation for its efficacy in reducing bacterial pulmonary infections.

Without question, alveolar macrophages lavaged from cigarette smokers differ from those recovered from nonsmokers. Many parameters vary: absolute quantity (7, 26, 34-36), cytoplasmic inclusions altering morphology (34, 36-38), quantity of intracellular enzymes (35, 38), and higher resting glucose-energy requirements (34). However, phagocytosis of *Staphylococcus albus* (34) and heat-killed *Candida albicans* or *Aspergillus fumigatus* spores (39) has not been different. Recently, however, Yeager, Zimmet, and Schwartz (36) noted that pinocytosis of [^{14}C]sucrose was decreased in smokers' macrophages. In our studies, we did not find a noticeable difference in the uptake of IgG-opsonized *Pseudomonas* by alveolar macrophages obtained from smokers and nonsmokers (compare Figs. 1 and 2). It is possible that subtle differences were minimized because of our decision to culture the macrophages in vitro for at least 24 h before bacterial challenge. This routine was established because it was found that fresh glass-adherent alveolar macrophages (2-3 h after bronchial lavage) invariably formed cell surface rosettes with IgG-opsonized erythrocytes (EA) and did not phagocytose these immune complexes (21). It required 24-30 h of in vitro acclimization with our experimental culture conditions before erythrophagocytosis of EA complexes became maximal. Harris, Swenson, and Johnson (34) used freshly lavaged alveolar macro-

phages in phagocytosis studies with *Staphylococcus albus* mixed in autologous serum. They found that only small numbers of macrophages (18.5-23%) had phagocytized bacteria at the end of a 3-h incubation period. In contrast, Cohen and Cline (39) demonstrated that with prolonged in vitro culture for many days there was a progressive increase in phagocytic capacity of alveolar macrophages that was a function of increasing cell size. To get a high percentage of macrophages to ingest microorganisms for satisfactory phagocytic experiments, a period of cell adjustment to in vitro conditions is necessary. However, in this interval macrophages might dispel metabolic products that were perhaps toxic in the original alveolar environment; thus, initial differences in cellular function could be overlooked between smokers and other controls.

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REFERENCES

1. Green, G. M. 1970. The J. Burns Amberson Lecture—in defense of the lung. *Am. Rev. Respir. Dis.* **102**: 691–703.
2. Green, G. M. 1973. Alveolobronchiolar transport mechanisms. *Arch. Intern. Med.* **131**: 109–114.
3. Tillotson, J. R., and A. M. Lerner. 1968. Characteristics of nonbacteremic Pseudomonas pneumonia. *Ann. Int. Med.* **68**: 295–307.
4. Rose, H. D., M. G. Heckman, and J. D. Unger. 1973. *Pseudomonas aeruginosa* pneumonia in adults. *Am. Rev. Respir. Dis.* **107**: 416–422.
5. Graybill, J. R., L. W. Marshall, P. Charache, C. K. Wallace, and V. B. Melvin. 1973. Nosocomial pneumonia: a continuing major problem. *Am. Rev. Respir. Dis.* **108**: 1130–1140.
6. Pennington, J. E., H. Y. Reynolds, and P. P. Carbone. 1973. Pseudomonas pneumonia. A retroactive study of 36 cases. *Am. J. Med.* **55**: 155–160.
7. 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.
8. Hanks, J. H., and J. H. Wallace. 1958. Determination of cell viability. *Proc. Soc. Exp. Biol. Med.* **98**: 188–192.
9. Cohn, Z. A., and E. Weiner. 1963. The particulate hydrolases of macrophages. II. Biochemical and morphological response to particle ingestion. *J. Exp. Med.* **118**: 1009–1019.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
11. Crowder, J. G., M. W. Fisher, and A. White. 1972. Type-specific immunity in pseudomonas diseases. *J. Lab. Clin. Med.* **79**: 47–54.
12. Reynolds, H. Y., and J. S. Johnson. 1971. Structural units of canine serum and secretory immunoglobulin A. *Biochemistry.* **10**: 2821–2827.
13. Reynolds, H. Y., R. E. Thompson, and H. B. Devlin. 1974. Development of cellular and humoral immunity in the respiratory tract of rabbits to *Pseudomonas* lipopolysaccharide. *J. Clin. Invest.* **53**: 1351–1358.
14. Reynolds, H., M. Carta-Sorcini, and R. Mage. 1973. The immunoglobulins derived from a V_H-C_H recombinant rabbit and its normal relatives. I. Measurements of heavy chain molecular weights and allotypes of colostral IgA. *Immunochemistry.* **10**: 443–447.
15. Newcomb, R. W., D. Normansell, and D. R. Standworth. 1968. A structural study of human exocrine IgA globulin. *J. Immunol.* **101**: 905–914.
16. Little, J. R., and H. Donahue. 1968. Spectral properties of proteins and small molecules of immunological interest, in *Methods Immunol. Immunochem.* **2**: 343–364.
17. Fisher, M. W., H. B. Devlin, and F. J. Gnabasik. 1969. New immunotype schema for *Pseudomonas aeruginosa* based on protective antigens. *J. Bacteriol.* **98**: 835–836.
18. Eddie, D. S., M. L. Schulkind, and J. B. Robbins. 1971. The isolation and biologic activities of purified secretory IgA and IgG anti-*Salmonella typhimurium* "O" antibodies from rabbit intestinal fluid and colostrum. *J. Immunol.* **106**: 181–190.
19. 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.
20. Rabinovitch, M., and M. J. De Stefano. 1973. Particle recognition by cultivated macrophages. *J. Immunol.* **110**: 695–701.
21. 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.
22. Golde, D. W., T. N. Finley, and M. J. Cline. 1974. The pulmonary macrophage in acute leukemia. *N. Engl. J. Med.* **290**: 875–878.
23. Zipursky, A., E. J. Brown, and J. Bienenstock. 1973. Lack of opsonization potential of 11S human secretory γA. *Proc. Soc. Exp. Biol. Med.* **142**: 181–184.
24. Keimowitz, R. I. 1964. Immunoglobulins in normal human tracheobronchial washings. *J. Lab. Clin. Med.* **63**: 54–59.
25. Falk, G. A., A. J. Okinaka, and G. W. Siskind. 1972. Immunoglobulins in the bronchial washings of patients with chronic obstructive pulmonary disease. *Amer. Rev. Respir. Dis.* **105**: 14–21.
26. Sharp, P. M., G. A. Warr, and R. R. Martin. 1973. Effects of smoking on bronchial immunoglobulins. *Clin. Res.* **21**: 672. (Abstr.)
27. Ross, G. D., M. J. Polley, E. M. Rabellino, and H. M. Grey. 1973. Two different complement receptors on human lymphocytes. *J. Exp. Med.* **138**: 798–811.
28. Tourville, D. R., R. H. Adler, J. Bienenstock, and T. B. Tomasi, Jr. 1969. The human secretory immunoglobulin system: immunohistological localization of γA secretory "piece" and lactoferrin in normal human tissues. *J. Exp. Med.* **129**: 411–429.
29. Martinez-Tello, F. J., D. G. Braun, and W. A. Blanc. 1968. Immunoglobulin production in bronchial mucosa and bronchial lymph nodes, particularly in cystic fibrosis of the pancreas. *J. Immunol.* **101**: 989–1003.
30. VanFurth, R., and F. Aiuti. 1968. Immunoglobulin synthesis by tissues of the gastro-intestinal and respiratory tracts. *Protides Biol. Fluids, Proc. Colloq. Bruges.* **16**: 479–484.
31. Reynolds, H. Y., and R. E. Thompson. 1973. Pulmonary host defenses. I. Analysis of protein and lipids in bronchial secretions and antibody responses after vaccination

with *Pseudomonas aeruginosa*. *J. Immunol.* **111**: 358–368.
32. Fudenberg, H. H., R. A. Good, H. C. Goodman, W. Hitzig, H. G. Kunkel, I. M. Roitt, F. S. Rosen, D. S. Rowe, M. Seligmann, and J. R. Soothill. 1971. Primary immunodeficiencies: report of a World Health Organization committee. *Pediatrics.* **47**: 927–946.
33. Heiner, D. C., and L. Evans. 1967. Immunoglobulins and other proteins in commercial preparations of gammaglobulin. *J. Pediatr.* **70**: 820–827.
34. Harris, J. O., E. W. Swenson, and J. E. Johnson, III. 1970. Human alveolar macrophages: comparison of phagocytic ability, glucose utilization and ultrastructures in smokers and nonsmokers. *J. Clin. Invest.* **49**: 2086–2096.
35. Cantrell, E. T., G. A. Warr, D. L. Busbee, and R. R. Martin. 1973. Induction of aryl hydrocarbon hydroxylase in human pulmonary alveolar macrophages by cigarette smoking. *J. Clin. Invest.* **52**: 1881–1884.
36. Yeager, H., Jr., S. M. Zimmet, and S. L. Schwartz. 1974. Pinocytosis by human alveolar macrophages: comparison of smokers and nonsmokers. *J. Clin. Invest.* **54**: 247–251.
37. Pratt, S. A., M. H. Smith, A. J. Ladman, and T. N. Finley. 1971. The ultrastructure of alveolar macrophages from human cigarette smokers and nonsmokers. *Lab. Invest.* **24**: 331–338.
38. Martin, R. R. 1973. Altered morphology and increased acid hydrolase content of pulmonary macrophages from cigarette smokers. *Am. Rev. Respir. Dis.* **107**: 596–601.
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.