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Respiratory Carriage of Pseudomonas Aeruginosa by Cystic Fibrosis Patients: Use of the Nitroblue Tetrozolium Test as a Means of Monitoring Patient Status

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RESPIRATORY CARRIAGE OF *PSEUDOMONAS AERUGINOSA*

BY CYSTIC FIBROSIS PATIENTS: USE OF THE
NITROBLUE TETROZOLIUM TEST AS A MEANS OF MONITORING
PATIENT STATUS.

By

Paul Van Gorder

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Science.

October 1974
To my family,

to

Miriam and Pastor Lueking,

and to

Chris, Janice, Catherine, Scott, Dennis, Rosy, Terry, Deb, Tracey, Tim, Phil, Brian, Tim, Robert, Scott, Jennifer, Deanne, Steven, Karen, Maria, William, Lisa, Lauri, Scott, Keith, Glen, Mary Beth,

and Penny.
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INTRODUCTION

Cystic fibrosis is a genetically determined disease inherited as a Mendelian autosomal recessive trait. The nature and identity of this gene or possibly gene cluster is as yet unknown. Unlike such genetic conditions as Tay-Sachs disease, sickle cell anemia, or Lesch-Nyhan syndrome, which all have characterizable primary biochemical abnormalities, there is no such marker in cystic fibrosis. It has been suggested by Conneally et al. (10) that the incidence of expressed cystic fibrosis ranges between 1 in 1900 to 1 in 4000 live births from caucasian populations in several countries worldwide. According to Di Sant'Agnese (13) the overall incidence of homozygotes for cystic fibrosis in the total population of the United States is near 1 in 2000, with a heterozygote incidence of 5 percent in the overall population, making cystic fibrosis the most common fatal genetic disease among whites in this country. Di Sant'Agnese (13) has written an enlightening review of the symptomatology of the disease. Briefly, the disease produces a cluster of symptoms including failure to thrive, steatorrhea due to pancreatic achylia, increased sweat electrolytes, and abnormal mucus secretions in the lungs, upper respiratory tract, and nasopharynx resulting in nasal polyps and recurrent, eventually terminal pneumonias. In approximately 15% of newborn infants with cystic fibrosis, meconium ileus with or without other obstructive intestinal complications are found and require immediate surgical obviation. Currently, diagnosis of the disease is made on the basis of significantly increased sweat electrolytes in cystic fibrosis patients. This test is usually performed after other symptoms such as failure to thrive, chronic or recurrent pulmonary
difficulties, and steatorrhea have been observed in varying degrees. Fre­quenty these three most outstanding symptoms occur concommitantly. Rennert (44) has proposed some additional laboratory tests which yet require further investigation before being employed clinically. There are two tests mentioned in particular which seem to give most promise. These tests are theoretically able to ascertain the presence of heterozygote as well as homozygote conditions making them of potential use in genetic counselling. One test is based on the presence of ciliary dyskinetic factor present in the serum of both homozygotes and heterozygotes. The other test of potential usefulness is that which employs skin fibroblast tissue cultures and temporary lymphocyte cultures of homozygotes and heterozygotes of the cystic fibrosis gene. These cells show significantly under-methylated RNA when compared to control subjects.

Current treatment for patients with cystic fibrosis has been essentially supportive. The review of Di Sant'Agnese (13) described some of the palliative procedures employed in the treatment of cystic fibrosis. Doyle (18) gives a further description of current techniques of postural drainage and breathing exercises for patients with cystic fibrosis. Procedures employed include assuaging pulmonary problems by means of prophylactic and therapeutic anti­biotic therapy for controlling bacterial infections, and postural drainage to facilitate removal of thick and copious mucus secretions as well as nebulizer and mist tent therapy. Dietary management consists of high carbohydrate and protein diets with moderate fat intake. This is supplemented with porcine or other pancreatic enzyme preparations to aid digestion. Salt replacement therapy may be instituted either prophylactically, especially during hot summer weather, or therapeutically in the case of heat prostration. Surgical intervention is
not commonly employed, although it is necessary in such cases as meconium ileus or other intestinal obstructive complications. A further account of surgical treatment of pulmonary complications has been given by Holsclaw and Shwachman (31). They suggest surgical intervention, tube thoracostomy in the cases of severe degrees of pneumothorax or thoractomy with lung resection in the cases of bronchiectasis or frank lung abscess.

Research has been launched on several fronts of the problem concerning cystic fibrosis. This work can be broadly categorized under two basic headings: first, that which is involved with the cause of the disease including the identification of the essential genetic lesion(s) and its (their) immediate and primary biochemical and biophysical consequences, and second, that concerned with the secondary clinical manifestations, particularly in the area of bacterial respiratory infections and the relationship between cystic fibrosis patients and their peculiar bacterial flora.

Much research has been done in elucidating the basic and primary nature of this disease. McCombs (37) has written a substantial review of investigations conducted in this area of cystic fibrosis research both original and current. Major efforts here seem to be in four broad directions, the study of skin fibroblast and leukocyte tissue cultures from cystic fibrosis patients and carriers and their production of atypical biochemical substances, the study of the serum protein ciliary dyskinetic factor in both homozygotes and heterozygotes, the study of membrane transport abnormalities in cystic fibrosis patients, and the study of the composition and nature of mucus sols, saliva, and sweat as well as the physiology of their secretion.
Since the nature of this particular investigation is essentially concerned with the secondary clinical manifestations of this disease, namely, pulmonary infections with *Pseudomonas aeruginosa*, a more detailed literature review shall follow including the statement of purpose.
The purpose of this thesis is basically threefold. First, this project aims to determine the incidence, prevalence, and maintenance of Pseudomonas aeruginosa respiratory tract infections in cystic fibrosis patients by means of pyocine typing. Secondly, by means of nitro blue tetrazolium, stimulated and unstimulated tests of cystic fibrosis patients, severely and moderately to mildly affected, and adequate control subjects, it will be determined whether or not significant differences exist in test results between the three parameters mentioned above. Thirdly, an investigation is to be made into the possible effect of mucoid slime of certain strains of P. aeruginosa on antibiotic sensitivity.

As was suggested earlier in the introduction the most frequent ultimate cause of death among cystic fibrosis patients is pulmonary complications. Holsclaw (30) has described the nature and, briefly, the mechanism of these complications. Initially, such symptoms as air trapping and atelectasis are presented due to either partial or complete obstruction due to abnormally thick mucus secretions. During this time inflammatory changes take place from mechanical and bacterial sources, resulting in metaplasia of mucus secreting cells within the respiratory tract. This cycle feeds upon itself resulting in further damage. A complicated series of pathological changes then ensues at various and different times and places within the lung parenchyma, from bronchiectasis and frank lung-abscess to the ultimate events of emphysema, fibrosis, and cavitation. On the gross level, pneumothorax and staphylococcal empyema, and terminally, cor pulmonale and
pulmonary and cardiac failure occur.

Iacocca et al. (32) had observed, even twelve years ago, that there was a significantly increased prevalence in the isolation of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Haemophilus influenzae* from sputum and oro- and nasopharynges of cystic fibrosis patients. Higher incidences of *S. aureus* occurred in patients not treated with antibiotics (penicillins), while *H. influenzae* was found in their study to occur with similar frequency before and after treatment. The incidence of *P. aeruginosa* was found to increase with the age of the patient, while *S. aureus* remained stable in its prevalence. Their phage typing results with *S. aureus* demonstrated no particular prevalence of any one strain, but they observed the persistence of specific types in each patient. The English investigators, Mearns et al. (38) in a more recent study, have essentially supported the work of the previously mentioned investigators. However, they observed over the period of their study that the occurrence of *S. aureus* was decreasing overall, while that of *P. aeruginosa* was on the increase, as shown not only in the isolation rate, but in the serum precipitin titers as well. They had suggested that since the balance between the groups of patients with differing severities had not changed substantially, while the relative occurrence of staphylococci and pseudomonads had interchanged, that the two organisms have similar pathogenic significance. This may be reflective of the trend in recent years of the replacement of gram positive coccal infections by gram negative rods in nosocomial infections, as well as the advent of better antistaphylococcal antibiotic therapy, this latter observation made by Doggett and Harrison (15). Besides these factors, it has been noted in an excellent clinical laboratory
review on the genus *Pseudomonas* by Franklin and Franklin (21), that *P. aeruginosa* possesses extreme metabolic versatility, resistance to a wide variety of antibiotics and disinfectants, and tends to be distributed ubiquitously both inside and outside of the hospital environment. For these reasons, it is also suggested that patients whose body defense systems are in any way compromised are rendered more highly susceptible to infection and pathology by this agent. The work of Doggett and Harrison (15) has been able to suggest further implications from respiratory tract infection by *P. aeruginosa*. They demonstrated that serum precipitins against *P. aeruginosa* were found only after organisms of mucoid colonial morphology were isolable from the sputum of cystic fibrosis patients. Contrariwise, serum precipitins were not found against *P. aeruginosa* in patients colonized by typical, rough, non-mucoid pseudomonas. In these cases the test antigen was obtained from the patients own pseudomonad. It was noted that patients with both serum precipitins against mucoid *P. aeruginosa* as well as colonization by mucoid organisms themselves, generally have severe disease and a poor prognosis. This is contrasted with those patients with less severe disease who are colonized only by non-mucoid pseudomonas or not colonized at all. One of the major points in their study was that once a patient with cystic fibrosis has become infected and colonized with non-mucoid *P. aeruginosa*, conversion to or replacement by the mucoid form is almost inevitable. As well, once colonization by mucoid *P. aeruginosa* occurs, it is probably impossible to completely eradicate the carriage state. Finally, it was observed in their study that the slime from the atypical cystic fibrosis pseudomonad is different from that of the rarely encountered "natural" mucoid *P. aeruginosa*. 
There seems to be, then, a unique relationship between the cystic fibrosis patient's mucus and the alteration and adaptation made by this organism.

The subject of the atypical *P. aeruginosa* has been one of considerable interest, particularly with respect to the nature and production of the capsular slime material. The study of Schwarzmann and Boring (45) demonstrated a definite inhibition of polymorphonuclear leukocyte phagocytosis of certain bacterial species *in vitro* when the mixture was incubated with the atypical mucoid pseudomonas slime isolated from an organism isolated from a cystic fibrosis patient. This leads to the possible suggestion that this slime may well contribute to the overall lung pathology in cystic fibrosis patients infected by this organism. Doggett et al. (17) found a notable difference to exist between the capsular slime obtained from *P. aeruginosa* of cystic fibrosis origin and that of non-cystic fibrosis origin. The former demonstrated considerably higher viscosity as well as a marked insolubility in a mixture of benzene and ethanol, while the latter did not. Another point made in this study was that mucus obtained from various body secretions of cystic fibrosis patients exhibited similar properties and composition to that of the slime of the atypical *Pseudomonas*, once again strengthening the relationship of cystic fibrosis patients mucus to the unusual mucoid *P. aeruginosa*. The composition of this *Pseudomonas* slime polysaccharide was also partially characterized in this study. It was found to contain some hexoses and hexosamines, and two prominent unknown components. In another study conducted by Linker and Jones (33) the composition of the atypical *Pseudomonas* slime was further characterized. It was found to contain about five percent nucleic acid and traces of
hexoses, hexosamines, and proteins. The main component, however, was found to be a substance almost identical to alginic acid, determined both by means of chromatographic analysis and infra-red spectrophotometry.

One other unique feature of this atypical, mucoid Pseudomonas has been observed. Linker and Jones (33) in the previously mentioned study observed phage plaques in the atypical organisms which had reverted to non-mucoid forms. The implication made here was that of a relationship of "mucoidness" to phage infection. Martin (34) in a more recent study has discovered that rings of slime were noticed to form around phage plaques of certain strains of *P. aeruginosa*. Upon subculturing of these "slime" areas, the colonies obtained were mucoid in nature, and the character and properties of these variants were identical to those of fresh mucoid isolates of *P. aeruginosa* obtained from patients with serious chronic respiratory infections, especially those with cystic fibrosis. It seems, then, that maintenance of mucoidness depends upon the presence of phage in the lytic cycle in a state of what Martin (34) calls "pseudolysogeny". The suggestion which she has postulated is that persons susceptible to chronic infection by Pseudomonas are more liable to successive infections by a number of different strains of the organism, thereby increasing the chance of being colonized by a pseudomonad carrying that particular phage. Her postulate is consistent with the data obtained.

Since this may very well be the case, the next area of interest would be an epidemiological survey of *Pseudomonas aeruginosa*. Such an investigation might give insight into nature, and mechanism of infection by this organism as well, possibly into the mechanism of the mucoid "conversion".
Doggett and Harrison (14) briefly reported the findings in their serological typing of Pseudomonas strains obtained from the respiratory tract of cystic fibrosis patients when compared with organisms derived from non-cystic fibrosis sources. Serotype 1 and serotypes 1 and 6 were shown to occur simultaneously more frequently in cystic fibrosis patients than from other sources. The main emphasis of this article was the suggestion that a polyvalent vaccine be prepared and used in the prophylactic treatment of cystic fibrosis patients. Although no mention was made of that fact, it was assumed that the serological typing system used was that of Hahs (27) or a more recent modification thereof. Neither, here, was any mention made as to whether or not organisms obtained were mucoid or non-mucoid, nor was any suggestion made as to incidence, prevalence, or maintenance of the organism.

In a more recent article Diaz et al. (12) made a more intensive study of the serological groups of P. aeruginosa obtained from cystic fibrosis patients. It was found that the majority of P. aeruginosa isolates from cystic fibrosis patients, just as those isolates from all other sources, fell into three distinct serological groups. Notable also was the fact that pseudomonad serogroups could be isolated repeatedly over periods of many months, while some patients showed infections by other transient serological groups. One of the most salient features of this study was the demonstration of identical 0-antigens from mucoid and non-mucoid organisms obtained from the same patients. Another observation made by these investigators was one already mentioned earlier, that the increased incidence of mucoid Pseudomonas correlated closely with the severity of lung disease in cystic fibrosis patients.
Notable here was the fact that only a slight age difference existed between group severities. Likewise, this age difference of persons with cystic fibrosis was not an important factor in the specific immune response against P. aeruginosa. This response, occurring in about 71% of the patients studied, occurred according to the severity and nature of the infection. Antibody response was found to be more frequent among patients from whom Pseudomonas could be isolated on more than one occasion. Supporting the observation of multiple types of P. aeruginosa was the fact that serum antibody titers could be demonstrated in somewhat less than half of 17 patients with a specific, demonstrable antibody response. In a still more recent study Williams and Govan (50) gave further supporting evidence that mucoid and non-mucoid pseudomonads were different forms of the same strain. This was done by means of pyocine typing using the system and indicator strains of Gillies and Govan (22) and Govan and Gillies (25) while varying the cultural milieu of the test or unknown strains. Although equivocal results were obtained when pyocine was induced on solid cultures of both mucoid and non-mucoid pseudomonads obtained from the same patients, pyocine induced strains in liquid cultures demonstrated identical pyocine types of mucoid and non-mucoid organisms obtained from the same patient. The suggestion made for the explanation of this phenomenon was simply that the slime of the mucoid colonies, when organisms were cultured on solid media, did not permit adequate diffusion of pyocines into the medium in order for indicator inhibition to occur. However, in liquid-culture pyocine induction, the slime of the mucoid organisms was dispersed, allowing also for the dispersal of pyocines. This project proposes further investigation of the epidemiology of P. aeruginosa.
by pyocine typing in the respiratory tracts of cystic fibrosis patients.

The next area of interest concerns a recently developed hematological test which assesses neutrophil function. The nitroblue tetrazolium (NBT) test is a means of measuring NADH and NADPH oxidase activity in polymorphonuclear leukocytes (PMN's). Increased activity in these enzymes is brought about during periods of increased phagocytic activity by these cells. Patriarca et al. (42) have discussed this matter in some detail. This test has been shown to be of particular use in the demonstration of systemic bacterial infections except where neutrophils are not substantially involved in the overall course of the disease, e.g., tuberculosis. Four substantial reviews and discussions of the NBT test, its use and clinical significance are those of Gordon and Rowan (23), Silverman and Ryden (47), Matula and Paterson (36), and Gordon et al. (24). Since the NBT test has been put to clinical use, two groups of investigators have published their findings concerning the relationship of patients with cystic fibrosis and results of the NBT test. Sullivan and associates (48), using the unstimulated NBT test (the method employed which does not use exogenous agents to stimulate phagocytosis in the test system) reported that a significant correlation existed between increased NBT tests scores and presumed respiratory tract bacterial infections. Stable patients, on the other hand, demonstrated NBT test scores which fell within control limits. It was noted that NBT results could not be correlated with total white cell count, percentage of bands, temperature of the patient, or change in sputum bacterial flora. The germaine point made by these investigators was the contention that serial NBT testing could be used as a valuable adjunct in assessing response to therapy. A more recent
study conducted by Hill et al. (29) supported the results and contention of the previous investigators, that increased NBT test results correlated closely with active pulmonary infection in cystic fibrosis patients. The evidence presented by these workers also suggested that these pulmonary bacterial infections caused circulating neutrophils to become more responsive to chemotactic factors.

The NBT test may be modified in such a way as to reveal two aspects of neutrophil function simultaneously. Not only is the test capable of assessing the presence of systemically involved bacterial infections from patient blood samples, but when simultaneous samples are incubated with one additional component, a phagocytosis stimulating agent, such as latex particles or bacterial endotoxin, along with the dyes, current maximum potential NBT reduction by neutrophils can be observed. This is based on a percentage of total stimulated cells found to contain the reduced NBT dye in the form of dark blue-black formazan particles intracellularly. For a further discussion of the nature and purpose of this methodology see Windhorst et al. (51). It is essential to note at this point that the NBT test results are closely and directly correlated to intracellular killing capacity of neutrophils. This postulate was supported by Baehner and co-workers (4) and by Nathan et al. (39) in studies with leukocytes from patients with chronic granulomatous disease. One of the potential uses of the stimulated NBT test is one of long term monitoring of maximum neutrophil killing capacity as reflected in relative NBT dye reduction. It was recently reported by Alexander and associates (1) that periodic variation in neutrophilic killing capacity occurred in all human subjects tested. The method used was the quantitative
bacterial count of *S. aureus* killed after phagocytosis by neutrophils, and the establishment of a neutrophilic bactericidal index. This variation in neutrophilic killing appeared to be cyclic with a somewhat irregular periodicity of 14 to 24 days. In patients whose host defense was particularly compromised, it was found that the risk of sepsis was closely correlated to periods of low neutrophil bactericidal activity. Thus, although development of low killing capacity was not always accompanied by bacterial sepsis, sepsis always occurred during periods of a relatively high neutrophilic bactericidal index (low killing capacity). It was found, as well, in burned and immunosuppressed patients, that periods of low neutrophil killing activity were substantially lower than in control subjects. To date, one of the most significant applications of the stimulated NBT test has been the monitoring of killing capacity of the neutrophils in burned patients and the prediction of burn wound sepsis by NBT test results. In the study of Curreri et al. (11), it was conclusively demonstrated that burn wound sepsis, measured by serial tissue biopsy cultures of greater than $10^5$ organisms per gram of tissue, was preceded by a substantial drop of the NBT test value taken upon hospital admission for each patient. On the basis of a 50% drop in the NBT test value on admission, it was suggested that burn-wound sepsis was either present or imminent. One of the most salient features of this investigation was the observation that the occurrence of burn sepsis and decrease in admission NBT test value was not simultaneous, but in fact, significant decreases in NBT scores were observed to occur in several instances several days prior to the onset of wound sepsis. In other words, the stimulated NBT test could be a valuable "early-warning system" in the prediction of such a
bacterial infection. It is with this in mind that such a system may also be effective in predicting infections or exacerbations in the respiratory tracts of patients with cystic fibrosis, which are so very much a part of morbidity and mortality of the disease.

Finally, it was thought to be of interest to make a simple preliminary study on the possible differences between mucoid variants and their own non-mucoid revertants of \textit{P. aeruginosa} obtained from selected children with cystic fibrosis with respect to their antibiotic sensitivities. As was observed earlier, more severely afflicted cystic fibrosis patients tend to be colonized to a greater proportion by mucoid \textit{Pseudomonas}. One speculation made by this investigator concerned the possibility that mucoid slime may somehow confer a greater antibiotic resistance upon these organisms over and against rough, non-mucoid forms. Thus, this factor might have a possible role in the overall pulmonary pathology of the disease. M.R.W. Brown (8) has suggested in a recent communication that much research has shown that the ability of \textit{P. aeruginosa} to be relatively resistant to many antibiotics is by a mechanism of cell surface layer exclusion chiefly at the site of the cytoplasmic membrane. That is to say that the various antibiotics are not able to penetrate into the appropriate site of action. It was thought with this in mind, that the possibility may exist of mucoid slime conferring some additional antibiotic resistance to \textit{P. aeruginosa}.
MATERIALS AND METHODS

Selection of subjects.

Altogether, a total of 28 cystic fibrosis subjects were considered in this study, including 13 female and 15 male patients. The age range from the beginning of the time of study (April 1973) was between 4 months and 19 years of age, the mean age being 8.4 years. Eleven of these patients were on prophylactic maintenance dosages of various antibiotics. Seven patients of the total were hospitalized at least once during the one year period of the study. All patients were alive at the end of the study. On the basis of Schwachman test scores from the criteria of Shwachman and Kulczycki (45), the patients could be divided into three groups: mild disease (score 100-81), moderate disease (score 80-61), and severe disease (score 60 or below). Assessment of the severity of pulmonary disease (chest x-ray results and pulmonary function studies) was also made on a scale of 0 to 5 (0-1, asymptomatic or mild; 2-3, moderate; 4-5, severe). These evaluations were made by Daniel G. Cunningham, M.D. at Foster G. McGaw Hospital, Loyola University Medical Center - Outpatient Pediatrics Clinic. For NBT test controls, 6 healthy, normal controls were used ranging in age from the beginning of the NBT test study (October, 1973) between 2 years and 18 years, with a mean age of 10 years. It is well to note that of the 10 cystic fibrosis patients selected for the NBT test study the average age was approximately 10.5 years, the range being 1 1/2 years to 19 years.
Isolation and identification of organisms.

Specimens were taken from throat cultures, prior to which time the patients were requested to cough up sputum from the lungs. Culturettes (Scientific Products) were used. Specimens were streaked out the same day as collected onto 5% sheep blood trypticase soy agar (BBL), MacConkey's agar (Difco), and Pseudosel agar (BBL), and incubated for 18 hr at 37°C. After that time the plates were observed and 3 or 4 colonies which resembled *P. aeruginosa* in odor, colonial morphology, pigmentation and "stained-glass window" hemolysis on blood agar, according to the criteria of Finkelstein and Punyashithiti (19) and King (33) were isolated and subcultured. Organisms twice subcultured by streaking to insure adequate isolation, were then subjected to the tests necessary for the identification of *Pseudomonas aeruginosa*, including oxidative fermentation tests, malonate utilization, flagella number and placement, growth at 42°C, and pigmentation, all according to the criteria of King (33) and Weaver (49). Isolated and conclusively identified organisms were then maintained as stock cultures in cystine tryptose agar (CTA) medium (BBL), being transferred every 6 months. It was discovered during the course of the study that after about one week of maintenance in the CTA medium that the mucoid organisms would usually lose their mucoid character irreversibly. It was subsequently discovered that the way to maintain mucoidness was by means of quick freezing in 1 ml aliquots of sterile defibrinated sheep blood or by maintaining cultures on trypticase soy agar (Difco) plates or subculturing every 48 hr as suggested by Goto et al. (25).

Pyocine typing

Pyocine typing was carried out using the method recommended by Dr. Hans
Griebel, M.D., of the Hines V.A. Hospital, Hines, Illinois (personal communication). The indicator strains employed were those of Gillies and Govan (22), obtained from Dr. Hans Griebel through Mr. J. T. Nickerson of Hines V.A. Hospital, Hines, Illinois, and those of Govan and Gillies (26) were obtained from Dr. Margaret G. Heckman, M.D. of Wood V.A. Hospital, Milwaukee, Wisconsin. Trypticase soy broth (TSB) (Difco) cultures of both indicator strains and unknown strains to be typed were incubated at 37°C for 18 hr on a Junior Orbit Shaker (Lab-Line). Aliquots of 0.1 ml of each indicator strain were transferred each to a fresh sterile 10 ml TSB and incubated by shaker culture at 37°C for 4 hr. After that time a lawn of each indicator strain was spread onto an appropriately labelled TSA plate with sterile cotton tipped applicators and allowed to dry. In addition, 1 ml aliquots of each unknown pyocine producing strain were transferred to fresh sterile 14 ml TSB tubes and were then incubated by shaker culture at 37°C for 2 hr. After that time 1 ml of sterile mitomycin C (Sigma), at a concentration of 30 micrograms/ml, was aseptically added to each tube. After a further 3 hr incubation, cultures were killed by the addition of 2 ml of chloroform to each tube, and were shaken. Cultures were allowed to settle and the still turbid supernatant fluid was aseptically decanted into another smaller sterile culture tube and spun down in a clinical centrifuge (Clay-Adams, Co., Inc., New York, N.Y.) at the highest speed (6) for 15 min. A 1:10 dilution of sterile distilled water was then made of the clear supernatant. By means of a flamed inoculating loop of about 5 mm diameter, one drop of each culture dilution was applied to each of the indicator plates, flaming the loop after each application. The plates were allowed to dry and then incubated for 18 hr
at 37°C. Plates were subsequently observed and assessed for the occurrence of plaques on the site of drop application. Plaques were graded on a scale of 0 to 4+, 0 representing no inhibition and 4+ complete clearing. On the basis of the relative inhibitions of each indicator strain after at least two separate typing assays, the patterns were then recorded for each of the unknown isolates. These results were then correlated with the typing schemes of Gillies and Govan (22), Govan and Gillies (25) and also Heckman and co-workers (28).

Before each of the mucoid unknown strains was inhibited by the mitomycin C, loopfuls of each culture were spread each onto a TSA plate in order to assess whether or not each culture was indeed still mucoid.

NBT testing

NBT tests were carried out in the following manner. Using sterile lancets after swabbing the skin with isopropyl alcohol blood samples obtained by finger or toe stick, or by heel stick in infants, were collected in heparinized Natelson blood collecting capillary pipettes (Dade). The pipettes were gently inverted several times before plugging one end to insure mixing of blood with heparin. Venous blood samples were drawn with the use of 5 ml heparinized vacutainer tubes (Becton-Dickinson) using Jelco 20 gauge disposable needles and plastic sleeves (Johnson and Johnson). The blood samples were immediately immersed in an ice water bath (0°C), unless the samples could be analyzed within 10 min of drawing. All samples were processed within 2 hr of drawing. Each 100 µl sample of blood was mixed with 100 µl of a stock NBT solution composed of 0.2% nitroblue tetrazolium Grade III (Sigma) w/v in 0.2 M sodium phosphate buffer of pH 7.4 in a 2 ml
disposable, conical, plastic microbeaker (Scientific Products B2713-2) and labelled. Into a second beaker were placed identical aliquots of 100 µl of blood and 100 µl of NBT-buffer stock solution, but to this mixture was added 10 µl of Difco 0.81 latex particle solution (Difco). Each beaker was gently mixed by agitation and immediately removed to a 37°C incubator for 25 min with a subsequent incubation for 5 min at 25°C. A portion of the supernatant plasma was then removed with a capillary tube to concentrate the cells. Blood smears were then prepared on Clean, labelled glass slides and allowed to air dry. Slides were fixed with absolute methanol (Scientific Products) for 10 min and then stained in a solution composed of 1 part (dry) safranin 0 (Allied Chemicals), 40 parts glycerol, and 100 parts deionized water (w/v/v) for 10 min, and finally rinsed gently and allowed to dry.

Slides were then examined under 1000 X oil immersion using a Zeiss microscope. Only neutrophils of good, distinct morphology were counted. In unstimulated tests (no latex) 100 cells were counted. Cells with deposits of blue-black crystals of formazan were counted as positive, all others were counted as negative. In stimulated tests (with latex particles) 100 cells were counted and these were grouped into two categories, depending on the number of particles within the cell and the amount of formazan deposit: 1) cells containing 5 or more latex particles without dye reduction (negative) and 2) cells containing 5 or more latex particles with dye reduction (positive).

Antibiotic sensitivity testing

Antibiotic sensitivity testing was carried out by tube dilution method (41). Two mucoid strains and their respective non-mucoid revertants of
*P. aeruginosa* were employed in this study. The first, PZ M20, was obtained from a severely ill cystic fibrosis patient, and the second, KN M11, from a patient with mild disease. The antibiotics selected for use were reagent grade for laboratory use only. They were disodium carbenicillin (Roerig), tobramycin (Lilly), gentamycin sulfate (Schering) and sodium colistimethate (Warner-Chilcott). Sterile serial two-fold dilutions were made in brain heart infusion (BHI) broth (Difco) of each antibiotic beginning with 800 µg/ml disodium carbenicillin, 100 µg/ml tobramycin, 200 µg/ml gentamycin sulfate, and 12306 µg/ml sodium colistimethate. A total of 8 serial dilutions were made from the first tubes, while the final tenth tube served as a control containing no antibiotic. Equal volumes of a $10^{-4}$ dilution in BHI broth of a 37°C, 18 hr culture of each organism were added, to each appropriate tube, that is one culture for each series of 10 tubes for each of the 4 antibiotics. (Each of the $10^{-4}$ dilution inocula gave readings of nearly 0 absorbance when compared to appropriate controls, on a Spectronic 20 (Bausch and Lomb) spectrophotometer.) This effected a final 2-fold dilution of the antibiotics in each tube. Tubes were incubated at 37°C for 18 hr and then observed macroscopically for growth inhibition. The MIC was considered to be the concentration of antibiotic in the first tube showing no visible turbidity. After observation, loopfuls of the contents of the MIC tube and the following tube were plated out onto BHI agar to check for adequate purity and homogeneity of organism, as well as whether or not reversion of mucoid to non-mucoid form had taken place. The experiment was performed in triplicate for disodium carbenicillin and in duplicate for all other antibiotics.
The Limulus amebocyte lysate assay for endotoxin was performed on several lots of 5 ml heparinized vacutainer tubes (B/D-lots 8113927, 8113238, and 8113689), on one lot of Micro-Natelson blood collecting tubes (Sherwood - lot 36031), on one lot of Micro-Caraway blood collecting tubes (Sherwood - lot 25631), and finally on two lots of Natelson blood collecting pipettes (Dade - lots 197H and 197B), which were those used in the NBT test. The Limulus assays were graciously performed by J. Robinson, M.D. of Hines V.A. Hospital, Hines, Illinois. The method employed was that of J. Levin, M.D. of John Hopkins Hospital, Baltimore, Maryland. The estimates of the amount of endotoxin inside the tubes were expressed in a semiquantitative fashion on a scale from 0 to 4+. All other reagents used, when not specified, were obtained from Fisher Scientific.
RESULTS

Survey of patient status

An expression of overall patient status considered from the end of the study is to be found in Table 1. From these data, patients were assigned into three arbitrary groups based on Shwachman scores mentioned in the Materials and Methods section. With no significant exceptions, the severity of pulmonary status correlated well on an individual basis with the Shwachman Index (index of overall severity), thereby facilitating categorization. Most of the patients' overall conditions remained stable throughout the study, with only two exceptions. Patient DH demonstrated a marked improvement in her overall condition early in the study, allowing the change in assessment from severe status to moderate status to occur prior to participation in the NBT testing. Patient PZ demonstrated a more or less steady decline in overall status during the study, allowing for the status to change from severe to more severe. Patients hospitalized for pneumonia during this study were the following: TrH - once, TH - twice, DH - once, SI - 5 times, TS - once, PW - once and PZ - three times.

Results of the survey for P. aeruginosa

The results of this survey demonstrate the occurrence of P. aeruginosa among patients with cystic fibrosis. Incidence of infection by mucoid and non-mucoid organisms, and by different pyocine types is correlated with severity of disease. To begin, a compilation of all cultures obtained from the 28 cystic fibrosis patients was made (Table 2). Here is expressed the total number of cultures by source, e.g., throat, nose, sputum, etc., as well as the number of isolates from which P. aeruginosa was isolated. Note
TABLE 1

Survey of overall cystic fibrosis patient status.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Birth Date</th>
<th>Shwachman* Index</th>
<th>Average Pulmonary + Status 0 - 5**</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>F</td>
<td>7/20/71</td>
<td>96</td>
<td>0</td>
</tr>
<tr>
<td>SC</td>
<td>M</td>
<td>3/7/71</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td>GG</td>
<td>M</td>
<td>4/8/59</td>
<td>93</td>
<td>1</td>
</tr>
<tr>
<td>LC</td>
<td>F</td>
<td>7/2/59</td>
<td>92</td>
<td>0</td>
</tr>
<tr>
<td>CB</td>
<td>M</td>
<td>7/9/67</td>
<td>85</td>
<td>0</td>
</tr>
<tr>
<td>KN</td>
<td>M</td>
<td>5/2/61</td>
<td>82</td>
<td>3</td>
</tr>
<tr>
<td>JF</td>
<td>F</td>
<td>9/21/65</td>
<td>81</td>
<td>1</td>
</tr>
<tr>
<td>TM</td>
<td>M</td>
<td>5/6/67</td>
<td>81</td>
<td>1</td>
</tr>
<tr>
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<td>F</td>
<td>7/5/61</td>
<td>81</td>
<td>3</td>
</tr>
<tr>
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<td>F</td>
<td>7/12/65</td>
<td>79</td>
<td>2</td>
</tr>
<tr>
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<td>M</td>
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<td>77</td>
<td>3</td>
</tr>
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<td>M</td>
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<td>1</td>
</tr>
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</tr>
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<td>3</td>
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<td>72</td>
<td>1</td>
</tr>
<tr>
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</tr>
<tr>
<td>DR</td>
<td>M</td>
<td>10/16/72</td>
<td>71</td>
<td>2</td>
</tr>
<tr>
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<td>F</td>
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<td>68</td>
<td>3</td>
</tr>
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<td>0</td>
</tr>
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<td>3</td>
</tr>
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<td>Patient</td>
<td>Sex</td>
<td>Birth Date</td>
<td>Shwachman Index</td>
<td>Average Pulmonary Status 0 - 5 **</td>
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<tr>
<td>---------</td>
<td>-----</td>
<td>------------</td>
<td>----------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>RW</td>
<td>M</td>
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<td>65</td>
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</tr>
<tr>
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<td>M</td>
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<td>61</td>
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</tr>
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<td>M</td>
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<td>55</td>
<td>4</td>
</tr>
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<td>M</td>
<td>11/13/71</td>
<td>53</td>
<td>4</td>
</tr>
<tr>
<td>SI</td>
<td>M</td>
<td>4/3/72</td>
<td>52</td>
<td>3</td>
</tr>
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<td>TrH</td>
<td>F</td>
<td>4/26/57</td>
<td>44</td>
<td>5</td>
</tr>
<tr>
<td>PZ</td>
<td>F</td>
<td>7/23/65</td>
<td>30</td>
<td>5</td>
</tr>
</tbody>
</table>

** 0 - Normal  5 - Severe

* Score 81 - 100 Mild Disease
Score 61 - 80 Moderate
Score 60 or below Severe

+ Radiographic data only
TABLE 2

Total number of cultures obtained from cystic fibrosis patients reflecting prevalence of respiratory tract infection by \textit{P. aeruginosa}.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Date first culture performed</th>
<th>Source</th>
<th>Total no. cultures</th>
<th>No. positive \textit{Pseudomonas} isolates (M, N &amp; N, N)+</th>
</tr>
</thead>
<tbody>
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<td>CB</td>
<td>5/8/73</td>
<td>Throat</td>
<td>17</td>
<td>None</td>
</tr>
<tr>
<td>&quot;</td>
<td>11/6/73</td>
<td>Nose</td>
<td>4</td>
<td>None</td>
</tr>
<tr>
<td>JB</td>
<td>6/26/73</td>
<td>Throat</td>
<td>8</td>
<td>7N</td>
</tr>
<tr>
<td>&quot;</td>
<td>11/19/73</td>
<td>Nose</td>
<td>4</td>
<td>1 N</td>
</tr>
<tr>
<td>CC</td>
<td>9/25/73</td>
<td>Throat</td>
<td>1</td>
<td>1 M &amp; N</td>
</tr>
<tr>
<td>LC**</td>
<td>4/23/73</td>
<td>Throat</td>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td>SC</td>
<td>5/8/73</td>
<td>Throat</td>
<td>6</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>1/6/73</td>
<td>Nose</td>
<td>1</td>
<td>1 N</td>
</tr>
<tr>
<td>LF**</td>
<td>11/20/73</td>
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<td>None</td>
</tr>
<tr>
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<td>Nose</td>
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<td>&quot;</td>
</tr>
<tr>
<td>JF</td>
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<td>1 N</td>
</tr>
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<td>None</td>
</tr>
<tr>
<td>TrH</td>
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<td>11 M, 1 N &amp; M</td>
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<td>None</td>
</tr>
<tr>
<td>TH</td>
<td>5/8/73</td>
<td>Throat</td>
<td>14</td>
<td>13 M, 1 N &amp; M</td>
</tr>
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<td>2 M</td>
</tr>
<tr>
<td>&quot;</td>
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<td>Sputum</td>
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</tr>
<tr>
<td>DH</td>
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<td>Throat</td>
<td>14</td>
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</tr>
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<td>Throat</td>
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</tr>
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<td>&quot;</td>
<td>11/6/73</td>
<td>Nose</td>
<td>2</td>
<td>&quot;</td>
</tr>
<tr>
<td>SI</td>
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<td>Throat</td>
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<td>12 N</td>
</tr>
<tr>
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<td>2 N</td>
</tr>
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<td>3 N</td>
</tr>
<tr>
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<td>Nose</td>
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<td>None</td>
</tr>
<tr>
<td>KL</td>
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<td>3</td>
<td>2 M &amp; N, 1 M</td>
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<td>Nose</td>
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<td>2 M</td>
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<td>R/Ear</td>
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<td>Patients</td>
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<td>Source</td>
<td>Total no. cultures</td>
<td>No. positive Pseudomonas isolates (M, N &amp; N, N)*</td>
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<td>------------------------------</td>
<td>--------</td>
<td>-------------------</td>
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<td>&quot;</td>
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</tr>
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</tr>
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<td>PZ</td>
<td>4/25/73</td>
<td>Throat</td>
<td>13</td>
<td>12 M, 1 N &amp; M</td>
</tr>
<tr>
<td>&quot;</td>
<td>12/20/73</td>
<td>Nose</td>
<td>3</td>
<td>2 M, 1 N</td>
</tr>
</tbody>
</table>

** These patients had routinely shown negative throat and sputum cultures for P. aeruginosa prior to this study.

* M - mucoid
N - non-mucoid
M + N - both mucoid and non-mucoid
was made also of the occurrence of mucoid and non-mucoid organisms. Dates of first cultures were noted to indicate the length of participation of each patient in the study. A very significant proportion of these patients (71.5%) were found to be infected by *P. aeruginosa* (Table 3). A further concern was to break down the data on the occurrence of *Pseudomonas* in infected patients into the prevalence of both non-mucoid and mucoid organisms (Table 4). From the results presented thus far, an attempt was made to correlate the prevalence of *P. aeruginosa* with Shwachman scores (clinical severity of cystic fibrosis) (Table 5). Each horizontal row represents the total number of patients in that particular Shwachman score, rank, i.e., severe, moderate or mild. Each vertical column indicates the state of *P. aeruginosa* infection whether absent, present and demonstrating mucoid forms with or without non-mucoid forms, or present and demonstrating non-mucoid forms only. The percentage indicated next to each number represents the percentage of patients in each respective "severity" group which are infected or not by *P. aeruginosa*. The rest of the results of the *P. aeruginosa* survey are concerned with *Pseudomonas* pyocine typing of *P. aeruginosa* isolates and their occurrence in infected patients with cystic fibrosis, the relationship to type and colonial morphology the consistency and variability of pyocine typing results. All cystic fibrosis patients' *Pseudomonas* isolates which were pyocine typed, as well as the date each culture was isolated and the colonial morphology of the organism at the time of isolation, i.e., mucoid (M) or non-mucoid (N) are listed in Table 6. Then, the occurrence of each pyocine type or types in the case of multiple *P. aeruginosa* infection, with the number of patients harboring that (those) type(s), along with the ages of each patient was ranked (Table 7). The prevalence of mucoid and non-mucoid
TABLE 3

Prevalence of respiratory tract infection by *P. aeruginosa* in cystic fibrosis patients.

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected</td>
<td>8</td>
<td>28.5</td>
</tr>
<tr>
<td>Infected</td>
<td>20</td>
<td>71.5</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>100.0</td>
</tr>
</tbody>
</table>
TABLE 4

Prevalence of mucoid and non-mucoid strain respiratory tract infection of *P. aeruginosa* in cystic fibrosis patients.

<table>
<thead>
<tr>
<th>Infection by:</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-mucoid alone</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td>Mucoid and non-mucoid</td>
<td>9</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>Mucoid alone</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>100</td>
</tr>
</tbody>
</table>
### TABLE 5

**Correlation of Shwachman Index with the occurrence of mucoid and non-mucoid forms of *P. aeruginosa* in cystic fibrosis patients.**

<table>
<thead>
<tr>
<th>Shwachman index range</th>
<th>Relative severity of disease</th>
<th>Infected by non-mucoid forms only</th>
<th>Infected by mucoid forms*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 - 81</td>
<td>Mild</td>
<td>5 (62.5%)</td>
<td>2 (25%)</td>
<td>8 (100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-infected</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (25%)</td>
<td>1 (12.5%)</td>
<td>8 (100%)</td>
</tr>
<tr>
<td>80 - 61</td>
<td>Moderate</td>
<td>3 (20%)</td>
<td>5 (33%)</td>
<td>15 (100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Infected by non-mucoid forms only</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 (33%)</td>
<td>7 (47%)</td>
<td>15 (100%)</td>
</tr>
<tr>
<td>60 and below</td>
<td>Severe</td>
<td>0 (0%)</td>
<td>1 (20%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Infected by mucoid forms*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 (20%)</td>
<td>4 (80%)</td>
<td>5 (100%)</td>
</tr>
</tbody>
</table>

* This list includes both patients colonized by mucoid forms alone as well as those colonized with both mucoid and non-mucoid forms.
TABLE 6

Total number of pyocine typed positive *P. aeruginosa* throat culture isolates which were obtained from cystic fibrosis patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Date specimens originally obtained</th>
<th>Pyocine type&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>JB</td>
<td>11/19/73</td>
<td>2F (N), UT&lt;sup&gt;#&lt;/sup&gt; (N)</td>
</tr>
<tr>
<td></td>
<td>1/4/74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3/12/74</td>
<td>UT&lt;sup&gt;#&lt;/sup&gt; (N)</td>
</tr>
<tr>
<td></td>
<td>4/2/74</td>
<td>2F (N), UT&lt;sup&gt;#&lt;/sup&gt; (N)</td>
</tr>
<tr>
<td>CC</td>
<td>10/13/73</td>
<td>VA39C (M), 3E (N)</td>
</tr>
<tr>
<td>SC</td>
<td>4/26/74</td>
<td>1B (N)**</td>
</tr>
<tr>
<td>JF</td>
<td>1/23/74</td>
<td>UC&lt;sup&gt;##&lt;/sup&gt; (N)</td>
</tr>
<tr>
<td>TrH</td>
<td>6/12/74</td>
<td>VA39C (M)</td>
</tr>
<tr>
<td></td>
<td>7/10/74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7/31/74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9/11/74</td>
<td>VA39C (M), UT&lt;sup&gt;#&lt;/sup&gt; (M)</td>
</tr>
<tr>
<td></td>
<td>1/9/74</td>
<td></td>
</tr>
<tr>
<td>TH</td>
<td>5/8/73</td>
<td>3E (M)</td>
</tr>
<tr>
<td></td>
<td>6/19/73</td>
<td>3E (M), 3E (N)</td>
</tr>
<tr>
<td></td>
<td>11/20/73</td>
<td>3E (M)</td>
</tr>
<tr>
<td></td>
<td>1/4/74</td>
<td>3E (M)**, 3E (N)</td>
</tr>
<tr>
<td>DH</td>
<td>5/22/73</td>
<td>3E (M)</td>
</tr>
<tr>
<td></td>
<td>8/3/73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9/18/73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11/20/73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3/12/74</td>
<td></td>
</tr>
<tr>
<td>SI</td>
<td>4/19/73</td>
<td>3E (N)</td>
</tr>
<tr>
<td></td>
<td>10/8/73</td>
<td>1B (N)</td>
</tr>
<tr>
<td></td>
<td>11/20/73</td>
<td>LB (N), UT&lt;sup&gt;#&lt;/sup&gt; (N)</td>
</tr>
<tr>
<td></td>
<td>1/4/74</td>
<td>3E (N)</td>
</tr>
<tr>
<td></td>
<td>1/29/74</td>
<td>1B (N), 3E (N)</td>
</tr>
<tr>
<td>SK</td>
<td>6/19/74</td>
<td>UT&lt;sup&gt;#&lt;/sup&gt; (N)</td>
</tr>
<tr>
<td></td>
<td>6/25/74</td>
<td></td>
</tr>
<tr>
<td>KL</td>
<td>8/28/73</td>
<td>2F (M), 2F (N)</td>
</tr>
<tr>
<td></td>
<td>11/20/73</td>
<td>3E (M), 3E (N)</td>
</tr>
<tr>
<td></td>
<td>12/26/73</td>
<td>3E (M)</td>
</tr>
<tr>
<td>Patient</td>
<td>Date specimens eventually obtained</td>
<td>Pyocine type†</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>KN</td>
<td>5/22/73 UT# (M), UT# (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10/10/73 UT# (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4/2/73 UT (M), UT# (N)</td>
<td></td>
</tr>
<tr>
<td>MBQ</td>
<td>5/8/73 UT# (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/5/74</td>
<td></td>
</tr>
<tr>
<td>DR</td>
<td>6/25/73 1B (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8/20/73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10/10/73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/9/74</td>
<td></td>
</tr>
<tr>
<td>TS</td>
<td>11/20/73 UT# (M)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12/19/73</td>
<td></td>
</tr>
<tr>
<td>MT</td>
<td>11/20/73 1C (N)</td>
<td></td>
</tr>
<tr>
<td>PW</td>
<td>6/4/73 3E (M), 3E (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10/10/73 3E (M)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12/19/73 3E (M)</td>
<td></td>
</tr>
<tr>
<td>RW</td>
<td>4/10/73 1B (N), 3E (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5/8/73 3E (M)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6/19/73 3E (M), 3E (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10/30/73 3E (M), 3E (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12/26/73 3E (M), 3E (N)</td>
<td></td>
</tr>
<tr>
<td>RMW</td>
<td>6/5/73 UT# (M), UT# (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9/25/73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12/26/73 UT# (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3/1/74 UT# (M), UT# (N)</td>
<td></td>
</tr>
<tr>
<td>BW</td>
<td>6/5/73 1B (N), UT# (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7/24/73 1B (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12/27/73 3E (N)</td>
<td></td>
</tr>
<tr>
<td>PZ</td>
<td>4/25/73 UC## (M)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6/19/73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8/28/73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12/26/73 UC## (M), UC## (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/4/74 UC## (M)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4/2/74 UC## (M), UC## (N), 3E (M)</td>
<td></td>
</tr>
</tbody>
</table>

† M - mucoid N - non-mucoid
# UT - untypable
** Nasal isolates included
## UC - unclassifiable
TABLE 7

Carriage of single and multiple pyocine types of \textit{P. aeruginosa} in respiratory tract infections of patients with cystic fibrosis.

<table>
<thead>
<tr>
<th>Pyocine Type(s)</th>
<th>Number of Patients</th>
<th>*Ages of Patients in each group</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT$^+$</td>
<td>5</td>
<td>2.5 yr, 2 (13 yr), 8 yr, 18 yr</td>
</tr>
<tr>
<td>3E</td>
<td>3</td>
<td>9 yr, 16 yr, 20 yr</td>
</tr>
<tr>
<td>1B</td>
<td>2</td>
<td>1.5 yr, 3 yr</td>
</tr>
<tr>
<td>1B, 3E, UT$^+$</td>
<td>2</td>
<td>2 yr, 2.5 yr</td>
</tr>
<tr>
<td>1B, 3E</td>
<td>1</td>
<td>5 yr</td>
</tr>
<tr>
<td>1C</td>
<td>1</td>
<td>15 yr</td>
</tr>
<tr>
<td>2F, 3E</td>
<td>1</td>
<td>12 yr</td>
</tr>
<tr>
<td>2F, UT$^+$</td>
<td>1</td>
<td>9 yr</td>
</tr>
<tr>
<td>VA39C, UT$^+$</td>
<td>1</td>
<td>17 yr</td>
</tr>
<tr>
<td>VA39C, 3E</td>
<td>1</td>
<td>8 yr</td>
</tr>
<tr>
<td>UC**, 3E</td>
<td>1</td>
<td>9 yr</td>
</tr>
<tr>
<td>UC**</td>
<td>1</td>
<td>8 yr</td>
</tr>
</tbody>
</table>

TOTAL 20

* Calculated from the end of the study.
** Unclassifiable.
+ Untypable.
colonial morphologies of original \textit{Pseudomonas} isolates was then correlated to each of the respective strains' pyocine types (Table 8). In this table, too, it is to be noted that the totals for each pyocine type are obtained by including organisms isolated from each patient regardless of the source of isolation, e.g., throat culture, sputum, nose, etc. Correlating the above data, a number and percentage distribution of each pyocine type among infected patients is shown (Table 9). In the final two tables of this section, an example of inherent variability is shown in serial pyocine typing in one strain (SI 8) of a clinical isolate of \textit{P. aeruginosa} (Table 10). These variable isolates were obtainable from only 2 of the 20 infected patients. A more representative example of consistent serial pyocine typing results is shown using the single isolate (DR 8) (Table 11). This reproducibility was more characteristic of the rest of the typable \textit{P. aeruginosa} isolates.

Nitroblue tetrazolium (NBT) testing

Stimulated and unstimulated nitroblue tetrazolium (NBT) testing was performed serially on 16 subjects including both cystic fibrosis patients and control subjects. These results are expressed both graphically as serial test results obtained over a period of time (Fig. 1-3) and also as average NBT test results among severe and moderate to mild cystic fibrosis patient categories and control subjects. All NBT test results indicated in Fig. 1-3 were obtained using capillary blood samples only. All extra pertinent information is indicated on each graph at the time of its occurrence. The serial NBT test results of cystic fibrosis patients falling in the severely affected range (Shwachman scores of 60 or less) are shown
TABLE 8

The relationship of pyocine type to mucoidness or non-mucoidness of

*P. aeruginosa* obtained from cystic fibrosis patients.

<table>
<thead>
<tr>
<th>Pyocine type</th>
<th>Mucoid</th>
<th>Non-mucoid</th>
<th>Total+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B</td>
<td>--</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>1C</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2F</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3E</td>
<td>5</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>VA39C</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>UC</em></td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>UT</strong></td>
<td>4</td>
<td>7</td>
<td>11</td>
</tr>
</tbody>
</table>

*Unclassifiable pattern of inhibition (Inhibition of type E only).

**Untypable on the basis of no inhibition of any indicator strains.

+Totals for each type are obtained by taking the overall occurrence of
each type from individual patients regardless of source.
TABLE 9

Occurrence of each pyocine type with regard to the total number of pyocine-typed isolates obtained from the 20 infected cystic fibrosis patients.

<table>
<thead>
<tr>
<th>Pyocine type</th>
<th>Number of isolates for each pyocine type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
</tr>
<tr>
<td>UT⁺</td>
<td>9</td>
</tr>
<tr>
<td>3E</td>
<td>9</td>
</tr>
<tr>
<td>1B</td>
<td>5</td>
</tr>
<tr>
<td>2F</td>
<td>2</td>
</tr>
<tr>
<td>UC++</td>
<td>2</td>
</tr>
<tr>
<td>VA39C</td>
<td>2</td>
</tr>
<tr>
<td>IC</td>
<td>1</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>*<em>30</em></td>
</tr>
</tbody>
</table>

* The discrepancy between this number and that of the total number of patients (20) lies in the fact that 6 patients were infected with 2 different strains each, and 2 other patients were infected with 3 strains each.

⁺ UT - untypable.

++ UC - unclassifiable.
TABLE 10

Example of the variability of pyocine typing results in repeated trials using the same clinical isolate of *P. aeruginosa* designated SI 8.

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Relative strength and pattern of indicator inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E1  E2  E3  E4  E5  E6  E7  E8  6A  6B  6C  6D  6E</td>
</tr>
<tr>
<td>1</td>
<td>2.5  4   2.5  2  3.5  0   2.7  0   0   4   4   2.5  2.5</td>
</tr>
<tr>
<td>2</td>
<td>4    4   4    1.5  4   0  4    0   0   4   4   0   4</td>
</tr>
<tr>
<td>3</td>
<td>4    4   4    3    4   0  4    4   1   4   4   2   4</td>
</tr>
<tr>
<td>4</td>
<td>4    4   2.5  0   3    0  3    3   0   4   4   2   4</td>
</tr>
<tr>
<td>5</td>
<td>4    4   2    1    4   0  4    3   0   4   4   2   4</td>
</tr>
</tbody>
</table>

Average results  3.7  4.0  3.0  1.5  3.7  0  3.5  2.0  0.2  4.0  4.0  1.7  3.7

+  +  +  -  +  -  +  -  -  +  +  -  +

These relative average results correspond to the Gillies and Govan pyocine types E3, having selected, somewhat arbitrarily, any average value of 2.0 or less as a negative result.

*0 indicates no inhibition, 4 indicates complete inhibition.*
TABLE 11

Example of consistent pyocine typing results in repeated trials using the same clinical isolate of *P. aeruginosa* designated DR8.

<table>
<thead>
<tr>
<th>Trial number</th>
<th>* Relative strength and pattern of indicator inhibition.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E1 E2 E3 E4 E5 E6 E7 E8 6A 6B 6C 6D 6E</td>
</tr>
<tr>
<td>1</td>
<td>2 4 2.5 3 3 - 2 4 - 3.5 2 3.5 3</td>
</tr>
<tr>
<td>2</td>
<td>4 4 3 4 2.5 - 4 3.5 - 4 2.5 4 3</td>
</tr>
<tr>
<td>3</td>
<td>4 4 4 4 4 - 4 4 - 4 3 4 3</td>
</tr>
<tr>
<td>4</td>
<td>4 3.5 3 4 4 - 4 3 - 4 2.5 4 3</td>
</tr>
<tr>
<td>Average</td>
<td>3.5 3.9 3.1 3.8 3.8 - 3.5 3.6 - 3.9 2.5 3.9 3.0</td>
</tr>
<tr>
<td>results</td>
<td>+ + + + + - + + - + + + + +</td>
</tr>
</tbody>
</table>

These relative average results correspond to the Gillies and Govan pyocine type 1B.

* 0 indicates no inhibition, 4 indicates complete inhibition.
Fig. 1. Serial stimulated and unstimulated NBT test results of four subjects (a - d) with severe cystic fibrosis disease.
Figure 1c
TrH-CF patient 9 - 16 yrs.
Stimulated NBT test ○
Unstimulated NBT test ●

Figure 1d
SI-CF patient 0 - 21 mo
Stimulated NBT test ○
Unstimulated NBT test ●

Hospital admission 12/17/73
Discharged 1/6/74
Fig. 2. Serial stimulated and unstimulated NBT test results of six subjects (a - f) with moderate to mild cystic fibrosis disease.

Figure 2a
CB-CF patient ♀ - 6 yr.
Stimulated NBT test o
Unstimulated NBT test •

Figure 2b
MI-CF patient ♀ - 14 yrs.
Stimulated NBT test o
Unstimulated NBT test •
Figure 2c

DH-CF patient - 0 - 20 yrs.
Stimulated NBT test - o
Unstimulated NBT test - •
Hospitalization for acute cholangitis

Figure 2d

DR-CF patient 0 - 15 months
Stimulated NBT test - o
Unstimulated NBT test - •
Figure 2e
RMW-CF patient 9 - 12 yrs.
Stimulated NBT test ○
Unstimulated NBT test ●

Figure 2f
PW-CF patient 0 - 8 yrs.
Stimulated NBT test ○
Unstimulated NBT test ●
Fig. 3. Serial stimulated and unstimulated test results of six control subjects.
Figure 3c
GG-Control ♀ - 14 yrs
Stimulated NBT test ○
Unstimulated NBT test ●

Time (in weeks)

Figure 3d
ToH-Control ♀ - 9 yrs
Stimulated NBT test ○
Unstimulated NBT test ●

Time (in weeks)
Figure 3e

DT-Control ♂ - 5 yrs.
Stimulated NBT test ○
Unstimulated NBT test ●

Figure 3f

KH-Control ♂ - 2 yrs.
Stimulated NBT test ○
Unstimulated NBT test ●

Slight respiratory infection
in Fig. 1, while the results of the moderately to mildly affected group of cystic fibrosis patients (Shwachman scores of 61 or greater) are presented in Fig. 2. The NBT test results of the control subjects are shown in Fig. 3. Subjects TrH (Fig. 3d) and KH (Fig. 3f) are half-siblings of different cystic fibrosis patients. A compilation of all unstimulated and stimulated NBT test results are shown in Fig. 4 and 5, respectively. These results include not only those patients who participated in the serial NBT test study (Fig. 1-3), but include other subjects on whom occasionally NBT tests were performed. Z-score tests were used for the statistical analysis of the NBT test scores of Fig. 4 and 5, instead of the Student's T test because of the size of each sample. These results are presented in Table 12. In it are compared the control groups and two cystic fibrosis patient groups according to their respective stimulated or unstimulated NBT test result category. Differences between simultaneously drawn capillary and venous blood stimulated NBT tests were then compared (Fig. 6). In Fig. 6a blood from cystic fibrosis patient TH (Shwachman score 55) was drawn on two separate occasions one week apart and compared (Trials 1 and 2). In Fig. 6b blood from a control (non-cystic fibrosis) subject, BZ, was employed as above. The effects of two different pre-incubation temperatures on NBT test results was shown (Fig. 7). Capillary blood samples from a control subject, PVG, were kept for various periods of time before mixing and incubation with the NBT dye solution and/or latex particles.

Limulus lysate-endotoxin assay results

The Limulus lysate-endotoxin assay, which was performed by passing 1 ml amounts of endotoxin free distilled water through each individual tube, showed a 4+ positive result for the presence of endotoxin in every tube. This is to
Figure 4

Unstimulated NBT test results of severe, and moderate to mild cystic fibrosis patients, and control subjects.

Severe cystic fibrosis patients - 5 patients total
Moderate to mild cystic fibrosis patients - 11 patients total
Control Subjects - 7 subjects total

* S.D. = Standard Deviation
Stimulated NBT test results of severe, and moderate to mild cystic fibrosis patients, and control subjects.

Severe Cystic fibrosis patients - 5 patients total

Moderate to mild cystic fibrosis patients - 11 patients total.

Control subjects 7 subjects total.

* S.D. = Standard Deviation
TABLE 12

Z-Score tests for assessing significance of correlation between the three separate averages each of stimulated and unstimulated NBT test values.

\[
Z = \frac{\bar{X}_1 - \bar{X}_2}{\frac{s^2_1}{N_1} + \frac{s^2_2}{N_2}} = \frac{\bar{X}_1 - \bar{X}_2}{\frac{s^2_1}{N_1} + \frac{s^2_2}{N_2}}
\]

<table>
<thead>
<tr>
<th>I (Severe CF)</th>
<th>II (Mod.-Mild CF)</th>
<th>III (Control)</th>
<th>A (Severe CF)</th>
<th>B (Mod.-Mild CF)</th>
<th>C (Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\bar{X})</td>
<td>49.2</td>
<td>33.4</td>
<td>12.7</td>
<td>82.1</td>
<td>81.0</td>
</tr>
<tr>
<td>(S)</td>
<td>27.0</td>
<td>21.8</td>
<td>15.0</td>
<td>34.3</td>
<td>14.7</td>
</tr>
<tr>
<td>(N)</td>
<td>37</td>
<td>42</td>
<td>31</td>
<td>37</td>
<td>42</td>
</tr>
</tbody>
</table>

AVERAGES COMPARED | Z-SCORES | SIGNIFICANCE
--- | --- | ---
I - II | 2.8 | Significant difference at 1% level.
I - III | 7.1 | Significant difference at 1% level.
II - III | 4.8 | Significant difference at 1% level.
A - B | 0.2 | No significance at 5% level.
A - C | 1.4 | No significance at 5% level.
B - C | 2.1 | Significant difference at 1% level.
Fig. 6. A comparison of stimulated capillary and venous blood NBT values in a subject (TH) with severe cystic fibrosis disease (6a) or in control subject (BZ) (6b). (Samples were drawn within five minutes of each other).
Fig. 7. Stimulated and unstimulated capillary blood NBT test values for samples preincubated at 34°C (7a) or 0°C (7b). U, unstimulated NBT value; S, stimulated NBT value; T, preincubation period.
say that these results roughly corresponded to the positive control of 1 µgm endotoxin/ml. A listing of these tubes once again is as follows: 3 tubes each of Lots number 8113927, 8113238, and 8113689 Becton-Dickinson 5 ml heparinized Vacutainer tubes; 5 each of 2 lots, 197H and 197B, of Natelson blood collecting pipettes (Dade); and 5 each of one lot, number 36031, of Micro-Natelson blood collecting tubes (Sherwood), and one lot, number 25631, of Micro-Caraway blood collecting tubes (Sherwood).

Antibiotic sensitivity testing

Antibiotic sensitivity testing results are to be found in Table 13. The minimum inhibitory concentration (MIC) of each antibiotic against the two mucoid strains of P. aeruginosa and their respective non-mucoid revertants is indicated in µgm/ml of each antibiotic. It is interesting to note that partial reversion from mucoid to non-mucoid occurred during the course of each trial only of strain KN M11 being tested against sodium colistimethate (Warner-Chilcott).
TABLE 13

Minimum inhibitory concentrations (MIC's)* of various antibiotics for mucoid strains of two clinical *P. aeruginosa* isolates KN M11 and PZ M20 and their respective non-mucoid revertants.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Trial Number</th>
<th>Mucoid KN M11</th>
<th>Non-mucoid KN M11</th>
<th>Mucoid PZ M20</th>
<th>Non-mucoid PZ M20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium carbenicillin</td>
<td>1</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>50</td>
<td>25</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>1</td>
<td>0.8</td>
<td>0.8</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Gentamicin sulfate</td>
<td>1</td>
<td>1.6</td>
<td>3.2</td>
<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Sodium colistimethate</td>
<td>1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
<td>0.25</td>
</tr>
</tbody>
</table>

* MIC's expressed as µg/ml.
DISCUSSION

The genetically determined disease cystic fibrosis is one whose complications include severe respiratory obstruction by presumably viscid mucus and subsequent bacterial infections of S. aureus and with ever increasing frequency, P. aeruginosa. It was in that interest that this study was undertaken, namely to follow the incidence and prevalence of P. aeruginosa by characteristic colonial morphologies (mucoid and non-mucoid) and by pyocine type, to observe the serial stimulated and unstimulated NBT test results in cystic fibrosis patients and control subjects, and finally to observe whether or not mucoidness of the colonial morphology of P. aeruginosa affected antibiotic susceptibility of that organism.

The 28 cystic fibrosis patients studied represent a heterogeneous group with regard to age, sex, and severity of overall status including pulmonary status (Table 2). Also, there is a definite correlation between overall patient status as measured by the Shwachman score and respiratory tract infection with P. aeruginosa (Tables 2-5). Of the 28 patients studied, 20 (71.5%) harbored pseudomonads (Table 3). Twelve of these 20 (60%) had organisms with mucoid colonial morphology (Table 4). It can readily be seen in Table 5, which correlates the severity of cystic fibrosis patients' status with the occurrence of P. aeruginosa of both mucoid and non-mucoid colonial morphologies, that the mildly affected group is predominantly not infected by any Pseudomonas (62.5%). Moderately affected patients show a wider distribution among the three infection status categories. Finally, the severely affected
patients demonstrate a remarkably high infection rate by mucoid *P. aeruginosa* (80%). These results are in basic agreement with those of Doggett and Harrison (15), whose findings demonstrated that patients with serum precipitins against *P. aeruginosa* and colonization by mucoid forms have a poorer prognosis than those patients with no serum precipitins and colonization by only non-mucoid organisms.

The results of the pyocine typing study are shown in Tables 6 through 9. Table 6 is a compilation of *Pseudomonas* pyocine types of all *P. aeruginosa* isolates which were tested. Although the distinction is not indicated in the table, identical *Pseudomonas* pyocine types were obtained from nose cultures as those obtained from throat cultures from patients TH (2 times, pyocine type 3E), SI (one time, pyocine type 3E), TS (2 times, pyocine type UT), and PZ (2 times, pyocine type UC). As can be seen from this table, the pyocine type of strains isolated from each patient remains fairly constant, even for patients who harbor more than one pyocine type, with the exception of patients KL and BW, whose pyocine types were entirely different from isolation to isolation. Among the rest of the cystic fibrosis patients who were colonized by multiple types, the different types could be isolated either serially, as in the example of patient SI, or concomitantly as in the cases of patients TrH and JB. These observations are supported also by the work of Diaz and Neter (12) in their study of the serogroups of *P. aeruginosa* in infected cystic fibrosis patients.

Two important observations were made during the present pyocine typing study. In Table 7, the serial pyocine typing patterns are illustrated for just one strain, SI 8, of a *P. aeruginosa* isolate. A great variability was
found not only in this one isolate, but in all isolates of pyocine type 3E obtained from patient SI. All other pyocine types obtained from all other infected patients displayed a much greater stability. Such an example is to be found in Table 8. The single strain DR8 was serially typed on four different occasions and on each typing the relative inhibitions of each respective indicator strain were virtually the same. Chadwick (9) has also observed this phenomenon, but by typing serial isolates from the same site in the same patient, rather than serially typing single isolates. His suggestion is simply that the same organism may in fact be producing pyocines which may somehow be more labile or unstable under slightly varying conditions or that perhaps the indicator susceptibility may also change during serial typing. Either or both of these factors may be involved in the variability of the patterns of inhibition. The present study tends to support these possible mechanisms more directly by demonstration of the varying results with the same organism.

The other important observation to be noted is that during the course of the serial pyocine typing of all mucoid isolates of *P. aeruginosa*, no change in the pyocine typing pattern of any isolate could be observed during conversion from the mucoid to the non-mucoid state. This observation was also supported to a certain extent by Williams and Govan (50) who observed that mucoid and non-mucoid pseudomonads isolated from the same cystic fibrosis patient were of identical pyocine types. They thought that these mucoid forms might merely be variants of the same non-mucoid strain. The demonstration in this current study of identical pyocine types in mucoid organisms and their own respective non-mucoid revertants, as well as in the originally non-mucoid clinical isolates further supports this contention.
Three pyocine types collectively predominated in the upper respiratory tract infections of cystic fibrosis patients in this study (Table 9). These types, UT, 3E, and 1B, occurred either alone, together, or in coexistence with other types in 18 (90%) of the 20 infected patients. These three types were found to be the predominant infecting strains, with the non-pyocine producing strains UT and 3E comprising 60% of the infecting strains (Table 10). With the exception of the somewhat higher percentage of type UT strains, the results of the present study are similar to those of other workers. In the results of their experience with pyocine typing of P. aeruginosa isolates in a midwestern Veterans Administration Hospital, Heckman et al. (28) found that type 1 constituted 52% of the total isolates, followed by an untypable strain (12%), type 10 (11%), type 3 (7%) and type 5 (3%). The rest of the strains consisted of miscellaneous types, variable types and new types not described previously by Gillies and Govan (22). In their study of P. aeruginosa infections in a general hospital, it was found by Baltch and Griffin (5) that 31% of the typable organisms were of type 1, type 5 comprised 12%, type 3, 10% and type 10, 8%. Of the total number of isolates (238), 21 (9%) were found to be untypable. Considering the vast proportion of pyocine type 1, the Govan and Gillies pyocine subtyping system was most useful in further differentiating that type in particular. In their study (25) types 1B, 1C and 1D comprised about 74% of the total type 1 strains, and are represented in their decreasing order of prevalence. It is well to note again that types 1B and 1C were the only two type 1 strains found, with type 1B heavily predominating.
One interesting observation made in the present study that has not previously been documented in the literature, has led to a suggestion as to the possible "order" of infection by different pyocine types of \textit{P. aeruginosa} in patients with cystic fibrosis. It was noted from the results of Table 9, that although not all very young patients (3 years or less) were infected with type 1B, this type was found to infect only those patients who were three years of age or less. Another fact was that none of the type 1B strains obtained from any of the infected patients were found to be mucoid (Table 9). Of the five patients infected by this type 1B, three of these (SI, RW, and BW) were infected by other strains as well. The cases of RW and BW are particularly interesting in that both of these patients were initially infected with the non-mucoid type 1B, with or without concomitant infection by other types. Through the course of this study, both of these patients developed infections by mucoid strains of \textit{P. aeruginosa}, and in the case of patient RW, the type 1B strain was probably completely eliminated in favor of the mucoid type 3E. This "elimination" phenomenon has not been observed in any other patients. However, indirect evidence suggests that pyocine type 1B cannot successfully compete with other strains of \textit{P. aeruginosa} which eventually convert to a mucoid form. Thus, no type 1B has been observed in older patients and this type is apparently unable to convert to the mucoid form (Table 11). Another possible explanation of this occurrence is one in which certain strains of \textit{P. aeruginosa} may produce pyocines active against other strains, thereby inhibiting their growth in simultaneous culture or infection. This postulate has yet to be further documented.
Since it has been demonstrated that a certain group of pyocine types tend to be the predominant infectious agents, the question arises as to the possibility of preparing polyvalent *Pseudomonas* vaccines for use in eliciting protective antibody response in cystic fibrosis patients. In the work of other investigators, particularly that of Alexander *et al.* (2) with burn patients, and that of Young *et al.* (52) with cancer patients, successful results have been obtained in producing effective protective immune response in these patients against *P. aeruginosa*. In both of these studies, the most dramatic results were the decrease in mortality due to the infection by *P. aeruginosa* rather than a decrease in the infection rate itself.

The results of serial NBT testing as stated previously in the Results section are to be found in Fig. 1-3. Of the first four figures (Fig. 1a-d), three represent patients who were hospitalized for pneumonia during the course of the NBT testing: PZ, TH, and SI. The points of hospital admission and discharge are indicated on each graph. Also indicated was the duration of a severe exacerbation of respiratory symptoms in patient TH. The graphs of these three patients are particularly interesting in light of the considerable lowering of unstimulated NBT test results during or following each hospital admission, when compared to each respective admission value. Except in the case of patient PZ (the most severely affected of all cystic fibrosis patients studied - Shwachman index 30), the stimulated values fell simultaneously with the unstimulated test results. These results correlate quite well with the improvement in overall condition of each patient during the hospitalization. The results of this study correlate well with those of Sullivan and associates (48) in their serial monitoring of unstimulated
NBT tests in hospitalized cystic fibrosis patients. Their suggestion was that serial monitoring by means of the NBT test on such hospitalized patients would be useful in adding to the criteria used in determining improvement in condition. The results of the present study support this contention. It was hoped in this study that predictions could be made of impending exacerbations in pulmonary symptoms by serial results of the stimulated NBT test, according to the study of Curreri et al. (11). This was found not to be possible for some technical reasons to be discussed later. In the remaining figures (2a-f and 3a-f) including the moderate-mild cystic fibrosis group (Fig. 2) and in the control group (Fig. 3), the only unusual event was the hospitalization of patient DH for acute abdominal complaints at the end of the study. She was released asymptomatic after one week. From an inspection of Fig. 1a and c and 2a-f and 3a-f, it can be noted that the periodic variations as suggested by the study of Curreri et al. (11) is not evident in these results. Overall, the unstimulated NBT test results in most of the cystic fibrosis patients (Fig. 1a, b, d; 2a, b, c, f) show either extremely variable results, or of those who don't, the results remain rather consistently high, i.e., values greater than 20% NBT positive PMNs. Certain control subjects also showed a variability in unstimulated NBT test results, especially in Fig. 3d and e. This will be accounted for later in the discussion. Fig. 4 demonstrates the total unstimulated NBT test results from all cystic fibrosis patients and control subjects. There is a distinct difference between the three different groups. There seems, therefore, to exist a valid correlation not only between increased unstimulated NBT test results with the events which occur in patients with cystic
fibrosis, but that the test results increase also with the severity of these events. These results, although somewhat higher overall, are consistent with the findings of Hill et al. (29) as well as those of Sullivan et al. (48). The average stimulated NBT test results are indicated in Fig. 5. A relatively significant difference in the averages existed only between all cystic fibrosis patients and control subjects, but not between the two different cystic fibrosis patient groups. It is also evident that from the high standard deviation indicated in Fig. 5 for the severely affected patients, that these results were more highly variable than in the other two groups. In comparison to the relatively smaller standard deviation of the moderate to mild cystic fibrosis patient group, the greater variability in the severely affected group may reflect the increased susceptibility of these patients to exacerbations of their pulmonary symptoms. As noted earlier, it was not possible to demonstrate the periodic variability in neutrophil function reflected in stimulated NBT test results. Also, there were more instances of unexpectedly high unstimulated NBT test results in control subjects. The most probable cause of these results rests with the test system and technique rather than with the subjects themselves.

During the course of the project it was ascertained that simultaneously drawn samples of heparinized venous and capillary blood showed a distinct difference in stimulated NBT test results. In Fig. 6 the results are shown of two trials each of simultaneously drawn capillary and venous blood from a cystic fibrosis patient and a control subject. In both subjects the venous blood samples showed definitely higher stimulated NBT test values. These results are consistent with those obtained in similar experiments conducted
by Björkstén (7). This indicates that venous blood samples probably represent a more accurate and truer index of maximum stimulation. A possible reason for this occurrence was the suggestion made by the above cited author (7) that certain interstitial tissue fluids obtained along with capillary drawn blood might, to a variable extent, inhibit neutrophilic NBT dye reduction. Another possibility became of concern. In the work of Benitez-Bribiesca (6), it was discovered that NBT dye reduction became markedly reduced in the presence of levels of heparin greater than 100 I.U. per ml of blood. The maximum amounts of heparin in either capillary tube blood or Vacutainer blood were 75 I.U. heparin/ml blood and 30 I.U. heparin/ml blood, respectively. The averages for capillary and venous blood samples however were 50 I.U./ml and 30 I.U./ml respectively. Therefore, the possible effect of heparin on suppressing dye reduction in the NBT tests could be discounted in this case. The lability of the NBT test was further demonstrated from the results illustrated in Fig. 7. At the higher pre-incubation temperature the unstimulated NBT test results increased after a period of time to render false high positive values. This may possibly be accounted for by an increased rate in some stimulatory degradation products in the blood samples. Another explanation is the possibility of an artifactual stimulation of the test by the presence of endotoxin in the blood collecting tubes. In fact from the positive results of the Limulus-lysate endotoxin assay on the heparinized glass blood collecting tubes and pipettes this may well be the case. As observed earlier, there were a number of instances of unpredictedly high unstimulated NBT test results in afebrile, healthy control subjects, in spite of taking the precaution of immersing the tubes in the ice water baths until assays were performed.
Although only very rough semiquantitative measurements can be made as to the actual amount of endotoxin present in each tube, by comparison with known standards, it seems that there was enough endotoxin present in each tube to produce a concentration of approximately 1 µgm/ml of (blood) sample within one order of magnitude in either direction. Endotoxin is often used, just as are latex particles, to produce an in vitro stimulation of phagocytosis. In the study of Matula and Paterson (36), endotoxin was added in varying amounts to the NBT test system to determine the threshold of stimulation. Unfortunately the threshold lies somewhere between the wide ranges of 0.5 µgm/ml (no stimulation) and 200 µgm/ml (high stimulation). In the stimulated NBT test system of Park (40), endotoxin is added to the blood dye solution mixture to a concentration of 10 µgm/ml. This means that in some of the tubes there is probably enough endotoxin present in certain of the blood collecting tubes to pass the "threshold of stimulation" and cause in vitro false high positive "unstimulated" NBT test. Although individual unstimulated NBT test results among healthy controls showed unpredicted high values in about 1 out of 6 total trials, the averages of all values for control subjects (13% NBT positive neutrophils) were similar to, although slightly higher than, those of other investigators using similar systems. Park et al. (41) found an average of 8.5% positive neutrophils from venous samples obtained from 30 healthy controls, while Fikrig and associates (20) using a method similar to that of Park et al. (41) found an average of 5% positive cells among 50 control subjects. The inaccuracy of some NBT test results in this study has made the interpretation of some of the serial test studies difficult. However, the average value
of NBT test results, both stimulated (12.7%) and unstimulated (73.7%) for control subjects gives a baseline for comparison with the NBT values obtained from cystic fibrosis patients.

In order for the combined use of the stimulated and unstimulated NBT test to be more effective and accurate, steps must be taken to correct all of the above mentioned problems. In the future use of the NBT test for any reason, the absolute prerequisite is the establishment of a single, well controlled test system.

From the results of the antibiotic sensitivity tests with all four antibiotics using the four strains tested, no significant differences exist between the antibiotic susceptibility of mucoid or non-mucoid organisms obtained from either of two patients studied: KN-mildly symptomatic (Shwachman score 82) and PZ-severely symptomatic (Shawchman score 30). It would seem most probable from these results, that the mucoid slime layer does not contribute substantially to the pathogenicity of mucoid P. aeruginosa merely by altering the organism's antibiotic sensitivity. This slime layer would seem, therefore, to be a loosely adherent, completely permeable substance incapable of contributing to the Pseudomonas cytoplasmic membrane's ability to exclude various antibiotics. The recent report by Anderson (3) further supports this contention. In this study he used gentamicin disc sensitivity tests on mucoid and non-mucoid isolates of P. aeruginosa from cystic fibrosis patients. His results on strains obtained both from throat and sputum cultures showed similar antibiograms of mucoid and non-mucoid isolates from their respective source.
SUMMARY

Twenty eight patients with cystic fibrosis were involved in studies of respiratory tract infection by Pseudomonas aeruginosa. The nitroblue tetrazolium (NBT) tests on neutrophil function, and the antibiotic sensitivity of mucoid isolates and their respective non-mucoid revertants of P. aeruginosa strains obtained from selected patients were also determined. In the study of the respiratory tract infections it was determined that P. aeruginosa pyocine types UT, 3E, and 1B predominated as infecting organisms, and that patients with more severe overall symptoms tended to be infected more often by mucoid than by non-mucoid strains. NBT test results showed a significant correlation between the events occurring in patients with cystic fibrosis and above normal unstimulated NBT test results. The results of the serial stimulated NBT tests were not able to predict exacerbations of pulmonary symptoms. The antibiotic sensitivity tests revealed no significant differences in antibiograms of mucoid isolates and their respective non-mucoid revertants using carbenicillin, gentamicin, tobramycin and sodium colistimethate.
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APPROVAL SHEET

The thesis submitted by Paul Van Gorder has been read and approved by the members of the Advisory Committee listed below.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

November 11, 1974

Date

Signature of Advisory Committee Director

Advisory Committee

1. Dr. H. J. Blumenthal
2. Dr. D. G. Cunningham
3. Dr. S. Silberman
4. Dr. F. A. Montiel