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Heterogeneity of Antibiotic Resistance in Mucoid Isolates of *Pseudomonas aeruginosa* Obtained from Cystic Fibrosis Patients: Role of Outer Membrane Proteins

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Mucoid *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients are very heterogeneous and include a class which is hypersusceptible to carbenicillin (minimum inhibitory concentration, ≤1 μg/ml). Hypersusceptible mucoid *P. aeruginosa* isolates were found in 12 of 22 cystic fibrosis patients examined. In cystic fibrosis patients having both resistant and hypersusceptible mucoid strains, 24 of 54 mucoid colonies obtained from a sputum sample were found to belong to the hypersusceptible class. In most instances, hypersusceptible and resistant strains isolated from the same sputum sample were indistinguishable, aside from their antibiotic susceptibilities, by classical methods. A particular pair of mucoid isolates (one hypersusceptible and one resistant) was chosen for further study. The hypersusceptibility was not limited to carbenicillin but was found to extend to other penicillins, tetracycline, and trimethoprim but not to the aminoglycosides gentamicin and tobramycin. The hypersusceptibility of the mucoid strain was found to be unrelated to amount or ability to synthesize alginate. The hypersusceptible strain was found to have two additional outer membrane proteins (32,000 and 25,000 daltons) as compared with the resistant strain. The 32,000-dalton protein, termed protein N1, was found to be correlated to the hypersusceptibility phenotype, as all spontaneous mutants of the hypersusceptible mucoid strain which were capable of growing in the presence of 50 μg of carbenicillin per ml had lost the 32,000-dalton outer membrane protein. The possible origins of the hypersusceptibility phenotype and the implications of the heterogeneity of mucoid *P. aeruginosa* in the pathogenesis of *P. aeruginosa* are discussed.

Pseudomonas aeruginosa is noted for its innate resistance to numerous antibiotics, with mucoid isolates of *P. aeruginosa* generally being somewhat more resistant to antibiotics and surfactants than nonmucoid isolates (7). Typically, the minimum inhibitory concentration of carbenicillin (carbenicillin is widely used in antipseudomonas therapy [12]) against *P. aeruginosa* isolated from various sources falls within the range of 25 to 50 μg/ml (4, 12). However, May and Ingold (18) reported the existence of a hypersusceptible class of *P. aeruginosa* isolates from the respiratory tract which were inhibited by 6 μg of carbenicillin per ml. Hypersusceptible strains of *P. aeruginosa* accounted for 33% of the 111 strains examined, and the incidence of hypersusceptibility differed little in mucoid strains as compared with nonmucoid strains. In a subsequent study, Berche et al. (4) examined the susceptibility of 47 mucoid and 71 nonmucoid *P. aeruginosa* isolates to 18 antibiotics. Both mucoid and nonmucoid strains could be divided into two separate classes, one class consisting of strains very susceptible to most antibiotics and a second class containing more resistant strains. The mucoid strains were divided almost equally between the two classes, 45 and 55%, respectively (4). The nonmucoid strains were much more homogeneous, as 89% of the strains belonged to the resistant class (4). Although the strains were isolated from a variety of clinical sources, the majority of the susceptible strains were obtained from sputum. We and other workers studying the association of *P. aeruginosa* with chronic debilitating respiratory infection in patients with cystic fibrosis (CF) have also reported the isolation of hypersusceptible *P. aeruginosa* and, in addition, the simultaneous isolation of hypersusceptible and more resistant strains from individual sputa; in most instances, hypersusceptible and resistant strains isolated from the same sputum sample belonged

to the same pyocin type or serotype (29, 31; J. R. W. Govan, J. A. M. Fyfe, K. Lam, and J. W. Costerton, J. Infect. Dis., in press; J. R. W. Govan and J. A. M. Fyfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, D45, p. 47; J. R. W. Govan, J. A. M. Fyfe, R. Irvin, and J. W. Costerton, Proc. Cong. Cystic Fibrosis 8th, Toronto, p. 18a, 1980). The basis of the hypersusceptibility of these strains has not been established.

Previously, antibiotic hypersusceptibility in gram-negative bacteria has been correlated with alterations in the outer membrane. In particular, abbreviation of the lipopolysaccharide has been correlated with both the loss of outer membrane proteins (1, 13, 30) and increased susceptibility to numerous antibiotics (21, 26). Hypersusceptibility to antibiotics has also been reported to occur when the lipopolysaccharide has been altered but when the outer membrane proteins have remained constant (5). Outer membrane permeability has also been found to be altered by changes in the outer membrane protein composition (2, 22, 27, 33). Thus, the susceptibility of the hypersusceptible mucoid *P. aeruginosa* strains to a wide range of antibiotics was examined to confirm that the hypersusceptibility was likely due to increased outer membrane permeability, and subsequently, the outer membrane composition was examined, and the hypersusceptibility was correlated to the presence of additional outer membrane proteins.

MATERIALS AND METHODS

Organisms. The strains of *P. aeruginosa* used in this study were isolated from the sputa of patients with CF who were attending the CF clinic at the Royal Hospital for Sick Children, Edinburgh, Scotland. All strains investigated were isolated and maintained on Pseudomonas Isolation Agar (PIA; Difco Laboratories) and identified as *P. aeruginosa* by the production of pyocyanin and oxidase and the ability to grow at 42°C. Approximately 100 colonies of *P. aeruginosa* from each sputum sample were investigated. The mucoid strains 492a and 492c were chosen as typical examples of isolates occurring simultaneously in individual CF sputa and exhibiting normal resistance to carbenicillin (492a) and hypersusceptibility to carbenicillin (492c). In this study, the term mucoid indicates variants which produced copious mucoid growth on agar medium within 24 h of incubation at 37°C and resembled *P. aeruginosa* colonial type 5 (24). Spontaneous nonmucoid revertants were obtained by subculture from cultures grown in nutrient broth (Oxoid no. 2 with 0.5% Oxoid yeast extract; Oxoid Ltd.) at 37°C without agitation. Only one revertant was chosen from each broth culture to avoid siblings. Spontaneous variants which could grow in the presence of 50 μg of carbenicillin per ml were obtained from hypersusceptible strain 492c by plating 10^7 cells on Diagnostic Sensitivity Test Agar (Oxoid) containing 50 μg of carbenicillin per ml and incubating at 37°C for 48 h.

Antimicrobial susceptibility. The antibiotics were incorporated into Diagnostic Sensitivity Test Agar, held at 45°C, dispensed in 20-ml volumes in plastic petri dishes, and used within 24 h. Inoculum was prepared by suspending in 1 ml of saline a single colony from a PIA plate incubated for 24 h at 37°C. This suspension was diluted 1/100 to give approximately 10^4 organisms per ml. The inoculum was applied with a Mast multipoint inoculator. After the plates had been incubated at 37°C for 18 h, the minimum inhibitory concentration was calculated as the minimum concentration of antibiotic which inhibited growth of the organism. Susceptibility to carbenicillin was also investigated with a broth dilution technique. The inoculum used contained 10^4 cells cultured as described below for the extraction of bacterial exopolysaccharide.

Exopolysaccharide extraction. Mucoid strains were grown in 1% peptone (Difco)-2% sodium gluconate overnight at 37°C in an orbital incubator (Gallemkampf) at 140 rpm. A 1% inoculum of the culture was introduced into fresh medium, and incubation was continued for 24 h. Extraction and characterization of the exopolysaccharide as bacterial alginate was as previously described (9).

Pyocin typing. The pyocin typing technique used was that described by Williams and Govan (35) and incorporated the revised scheme of Govan (8).

Outer membrane preparations. Outer membranes were prepared by a modification of the method of Mühlradt and Golecki (19). Harvested cells were initially treated with toluene (3% [vol/vol] in 0.1 M cacodylate buffer [pH 6.80]) for 3 h at room temperature to ensure accessibility of Triton X-100 to the cytoplasmic membrane. Toluene-treated cells were then exposed to 1% Triton X-100 [vol/vol in 10 mM tris(hydroxymethyl)aminomethane buffer (pH 7.00) containing 8 mM $MgSO_4$ and 20% sucrose] for 18 h at room temperature (11). The outer membrane and peptidoglycan were then washed three times with 0.1 M cacodylate buffer (pH 6.80), treated with 1 mg of deoxyribonuclease I (Sigma Chemical Co.)-0.5 mg of ribonuclease (Sigma)-1 mg of lysozyme per ml for 3 h at room temperature, washed again three times, and suspended in distilled H_2O at a final protein concentration of 5 mg/ml.

SDS-polyacrylamide gel electrophoresis. The sodium dodecyl sulfate (SDS)-discontinuous polyacrylamide gel electrophoresis system J 4179 of Neville and Glossman (20) was used for analysis of protein components. Samples contained 5 mg of protein per ml as determined by the method of Lowry et al. (15) with bovine serum albumin as the standard. Samples were normally solubilized by adding 10 mg of Na_2CO_3-40 mg of SDS-100 μl of 2-mercaptoethanol to 1 ml of sample and boiling for 20 min. Samples were occasionally solubilized in the above mixture at 60°C for 20 min, in the above mixture minus 2-mercaptoethanol for 20 min with boiling, or alternately by precipitating the protein with an equal volume of 15% (wt/vol) trichloroacetic acid, neutralizing with 0.1 M NaOH, and solubilizing by boiling with SDS-2 mercaptoethanol for 20 min (10). Solubilized samples (10 μl each) were applied to a 20-cm slab gel consisting of 11 or 14% acrylamide and 0.1% bis-acrylamide and electro-

phoresized with a constant current of 50 mA. Gels were subsequently stained with Coomassie brilliant blue overnight and destained electrophoretically. Molecular weight assignments were made as described by Weber and Osborn (32) with the Daltons Mark VI SDS molecular weight marker kit (Sigma).

Electron microscopy. Cultures for electron microscopy were grown on PIA overnight at 37°C. Single colonies were scraped off the surface and suspended in either 0.1 M cacodylate buffer (pH 6.80) containing 0.05% ruthenium red and 0.1% glutaraldehyde or cacodylate buffer containing only 0.1% glutaraldehyde. Samples were then processed as described previously except that they were not enrobed in agar (23). All specimens were examined with an AE1 801 electron microscope operating with an accelerating potential of 60 kV.

RESULTS

Bacteriological investigation of 33 sputum samples from 22 CF patients chronically infected with *P. aeruginosa* showed that 12 patients harbored hypersusceptible strains that were inhibited by 1 μg of carbenicillin per ml or less. In eight of these patients, strains of *P. aeruginosa* exhibiting normal resistance (i.e., inhibition by 20 to 80 μg of carbenicillin per ml) were isolated simultaneously from the same sputum sample. Hypersusceptible strains were found among mucoid strains (24 of 54 examined) and nonmucoid strains (11 of 24 examined). In contrast, no hypersusceptible *P. aeruginosa* strains were found in 216 strains obtained from a wide variety of clinical, but non-respiratory, sources in non-CF patients. Four CF patients were infected with mucoid *P. aeruginosa* belonging to both hypersusceptible and normal classes. In each patient, the normal and hypersusceptible strains belonged to the same pyocin type.

A typical pair of mucoid strains, 492a and 492c, isolated from the same sputum sample was chosen for further study. Both strains were shown to belong to pyocin type 1/b and were indistinguishable on the basis of colonial appearance. Strain 492a exhibited normal resistance to carbenicillin, whereas strain 492c represented the hypersusceptible class.

The difference in susceptibility of strains 492a and 492c towards carbenicillin was also observed with a broth dilution technique. Strain 492c was inhibited by 1 μg of carbenicillin per ml, whereas strain 492a was inhibited by 50 μg/ml, when the strains were grown in 1% peptone-2% sodium gluconate. The exopolysaccharide produced by both 492a and 492c grown on Diagnostic Sensitivity Test Agar or in 1% peptone-2% sodium gluconate was identified by infrared spectroscopy as an alginate-like polymer of mannuronic and guluronic acids. Strain 492c produced five times as much exopolysaccharide as strain 492a in 1% peptone-2% sodium gluconate (without carbenicillin) (Table 1). The hypersusceptibility of strain 492c was not directly associated with alginate production because six nonmucoid revertants from which no alginate could be detected were as susceptible to carbenicillin as strain 492c.

A further investigation was then made to determine if the hypersusceptibility of strain 492c extended to other antibiotics. Table 1 summarizes the minimum inhibitory concentrations of various antibiotics against strains 492a and 492c and illustrates that hypersusceptibility extended to other penicillins, tetracycline, and trimethoprim but not to the aminoglycosides gentamicin and tobramycin.

Isolates 492a and 492c produced copious amounts of ruthenium red-staining exopolysaccharide when grown on PIA or Diagnostic Sensitivity Test Agar (data not shown). Spontaneous mucoid mutants of strain 492c that grew in the presence of 50 μg of carbenicillin per ml also produced large amounts of ruthenium red-staining exopolysaccaride when grown on PIA (data not shown). Nonmucoid revertants obtained from isolate 492c produced no ruthenium red-staining exopolysaccharide when grown on PIA (data not shown) and yet were still susceptible to less than 1 μg of carbenicillin per ml. The nonmucoid revertant obtained from isolate 492a produced no ruthenium red-staining material when grown on PIA (data not shown) and was not hypersusceptible to carbenicillin. These morphological observations confirmed the mucoid phenotype which was originally assigned by the colony morphology of the strain on PIA.

TABLE 1. *In vitro susceptibility of two mucoid strains (492a and 492c) of P. aeruginosa isolated from the same CF sputum sample*[a]

Antibiotic	MIC (μg/ml) against strain:	
	492a	492c
Carbenicillin	40	0.4
Azlocillin	8	0.2
Piperacillin	6	0.2
Ticarcillin	8	0.3
Methicillin	200	4
Gentamicin	4	2.5
Tobramycin	0.7	0.7
Tetracycline	12	2
Trimethoprim	50	4

[a] Both strains belong to pyocin type 1/b. The amount of alginate obtained from each strain grown in 1% peptone (Difco)-2% sodium gluconate for 24 h at 37°C was 2.33 mg per mg of dry cells for 492a and 11.43 mg per mg of dry cells for 492c. Medium, Diagnostic Sensitivity Test Agar. Inoculum, 10^4 cells.

An examination of the outer membrane protein composition of strains 492a and 492c revealed the presence of two additional outer membrane proteins of 32,000 and 25,000 daltons in the outer membrane of strain 492c (Fig. 1). These additional outer membrane proteins did not appear to be heat modifiable or to contain disulfide bonds and did not appear to constitute a solubilization artifact (Fig. 2). Resistant mutants obtained from isolate 492c by selecion of mucoid colonies on Diagnostic Sensitivity Test Agar containing 50 μg of carbenicillin per ml lost the 32,000-dalton outer membrane protein but not the 25,000-dalton outer membrane protein (Fig. 3). The presence or absence of an extensive capsule had no effect on the outer membrane protein composition (data not shown).

DISCUSSION

This investigation of hypersusceptibility in mucoid *P. aeruginosa* might appear to contradict our previous research (9), in which we reported that mucoid strains exhibit enhanced resistance to antibiotics such as carbenicillin. The explanation is that mucoid *P. aeruginosa* strains are extremely heterogeneous. Although the association of mucoid *P. aeruginosa* with respiratory infection in CF patients has been known since the early report of Doggett (6), only recently has the heterogeneity of mucoid strains, in general and also within individual CF patients, been appreciated (29, 31; Govan and Fyfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, D45, p. 47; Govan et al., Proc. Cong. Cystic Fibrosis 8th, Toronto, p. 18a, 1980). Some mucoid strains of *P. aeruginosa* are demonstrably more resistant to penicillins and aminoglycosides than related nonmucoid strains. As a consequence, alginate-producing mucoid variants can be isolated readily in vitro from nonmucoid strains by selecting for enhanced resistance to carbenicillin or aminoglycosides (9). Individual isogenic variants, however, although sharing a common property of alginate production, may differ in other properties, such as the nutritional requirements necessary for alginate production. In our experience, it has not been uncommon to obtain from CF patients isolates that appear nonmucoid on blood agar but in reality are alginate-producing mutants if grown on PIA.

It should be emphasized also that nonmucoid revertants are heterogeneous. Some revertants retain the antibiotic resistance of the mucoid parent, whereas others are more susceptible (Govan et al., in press).

Our survey on the incidence of *P. aeruginosa* strains hypersusceptible to carbenicillin in CF patients confirmed the original report of the significant incidence of such strains in isolates from sputa (18). The incidence of hypersusceptible variants in association with more resistant strains in 55% of the 22 CF patients examined was slightly greater than the 38% incidence reported by Seale et al. (29). This could be due, however, to our intensive search involving 100 colonies from each sputum sample.

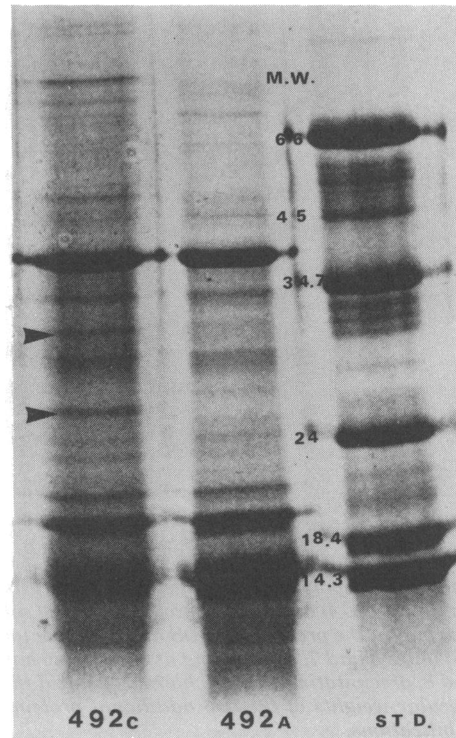

FIG. 1. *SDS-polyacrylamide gel electrophoresis (14% acrylamide gel) of outer membranes of strain 492a (mucoid resistant) and strain 492c, which has two additional proteins with apparent molecular weights of 32,000 and 25,000 (arrows). Molecular weight (M.W.) markers are 66,000 (66), 45,000 (45), 34,700 (34.7), 24,000 (24), 18,400 (18.4), and 14,300 (14.3). STD, Standard.*

Thomassen et al. (31) reported that individual CF sputa often produced one or more colonial types of *P. aeruginosa*: classic, rough, mucoid, gelatinous, dwarf, and enterobacter. Different colonial forms of *P. aeruginosa* isolated from the same sputum sample differed in antibiotic susceptibility, and it was suggested that susceptibility tests on isolates from CF patients should be performed on each colonial type. The results which we have reported in this investigation, exemplified by the similar colonial appearance of strains 492a and 492c, indicate that the heterogeneity of *P. aeruginosa* in CF patients de-

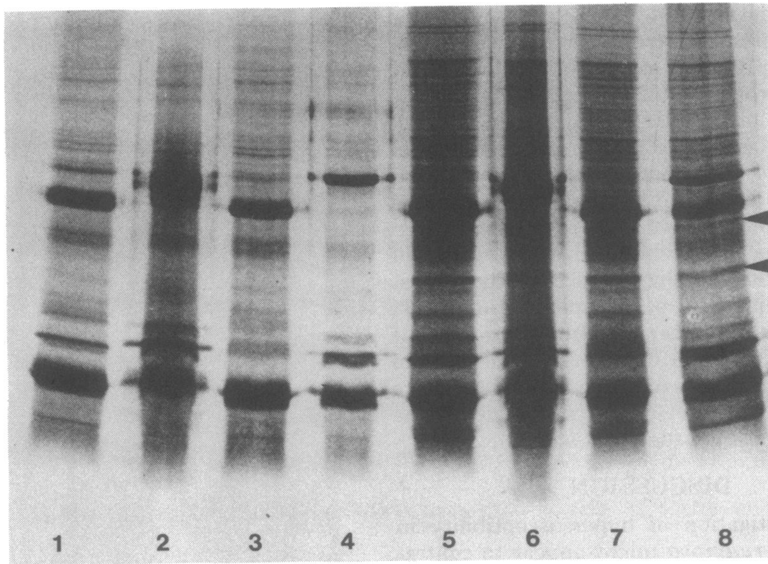

FIG. 2. *SDS-polyacrylamide gel electrophoresis (14% acrylamide gel) of outer membrane proteins of strain 492a (lanes 1 to 4) and strain 492c (lanes 5 to 8) solubilized by the following procedures: lanes 1 and 5, boiling for 20 min in the presence of SDS and 2-mercaptoethanol; lanes 2 and 6, boiling for 20 min in the presence of SDS; lanes 3 and 7, solubilizing at 60°C for 20 min in the presence of SDS and 2-mercaptoethanol; and lanes 4 and 8, precipitating with trichloroacetic acid then solubilizing as for lanes 1 and 5. Note that the apparent molecular weights of the two additional proteins (arrows) of strain 492c were not altered by the different solubilizations.*

mands even further consideration and examination of multiple examples of mucoid and nonmucoid colonial types.

The ability to act as donors in bacterial conjugation, to produce protease, and to agglutinate in the presence of homologous antisera is considerably diminished in mucoid variants as compared with their isogenic nonmucoid revertants. In contrast, the hypersusceptibility of strain 492c and other such strains was retained by nonmucoid revertants and not directly associated with alginate production. A close relationship, however, between the original mutation responsible for alginate production and hypersusceptibility cannot be discounted.

Although this investigation, together with those of May and Ingold (18) and Berche et al. (4), has shown hypersusceptible variants to be equally divided between mucoid and nonmucoid strains, it is not possible to distinguish between nonmucoid revertants which have arisen in vivo and the classic nonmucoid *P. aeruginosa* responsible for the original infection (unpublished observations). Thus, nonmucoid hypersusceptible strains isolated from CF patients could be revertants of a mucoid hypersusceptible variant and not true wild-type *P. aeruginosa*, as the hypersusceptibility phenotype is uncorrelated to mucoidy. Hypersusceptible strains are seldom encountered among nonmucoid strains of *P. aeruginosa* isolated from non-respiratory sources, but further investigation is necessary to establish the incidence of hypersusceptible strains among nonmucoid strains from respiratory sources in which no mucoid variants have been present.

The presence of hypersusceptible and normal variants within an individual sputum sample cannot be explained by simultaneous infection with more than one strain of *P. aeruginosa* or by cross-infection. No single pyocin type was responsible for the majority of normal or hypersusceptible strains investigated. Strains 492a and 492c, which were chosen for further study, not only belonged to the same pyocin type but were indistinguishable in colonial appearance from the uncommon pigment pyorubrin after incubation for 3 days at 37°C on PIA. Similarly, other pairs of resistant and hypersusceptible strains isolated from individual sputa appeared to be isogenic (unpublished data).

Increased susceptibility or hypersusceptibility to a wide range of antibiotics in gram-negative bacteria has generally been correlated to a cell envelope alteration, such as an abbreviation of the lipopolysaccharide (21, 26), a septation mutation (33), or an alteration in the lipopolysaccharide (5). As changes in outer membrane

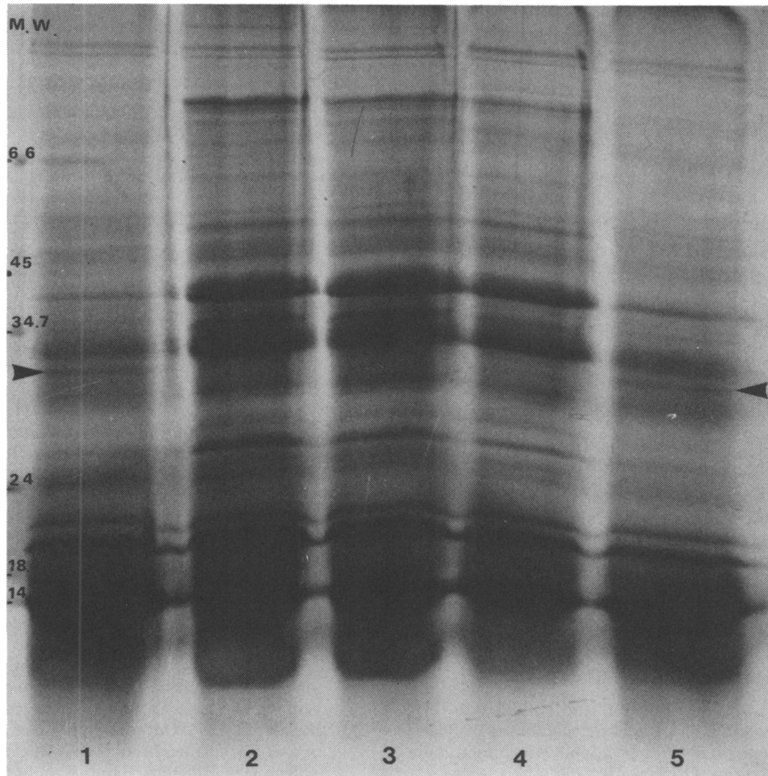

FIG. 3. *SDS-polyacrylamide gel electrophoresis (14% acrylamide gel) of outer membrane proteins of strain 492c (mucoid hypersusceptible) (lanes 1 and 5) and three mucoid resistant mutants (lanes 2, 3, and 4) obtained from strain 492c. Note that the mucoid resistant strains have lost the 32,000-dalton protein (arrows). See legend to Fig. 1 for explanation of numbers at left.*

permeability characteristics could be due to an alteration in the outer membrane protein composition, the outer membrane proteins of isolates 492a and 492c were compared.

The outer membrane protein compositions of isolates 492a and 492c are remarkably similar and differ only in the two additional outer membrane proteins with apparent molecular weights of 32,000 and 25,000 in isolate 492c (we propose to call the 32,000-dalton protein N1 and the 25,000-dalton protein N2 to conform with the nomenclature established by Hancock and Carey [10]) as compared with isolate 492a and in the relative abundance of a few outer membrane proteins (Fig. 1). Proteins N1 and N2 are not heat modifiable or 2-mercaptoethanol modifiable (Fig. 2), and thus protein N2 does not appear to be protein G (10). Protein N1 appears to be involved in the hypersusceptibility phenotype of isolate 492c, as all resistant mutants obtained from isolate 492c either completely lacked protein N1 or at least had a considerably reduced level of protein N1, whereas those outer membrane proteins of isolate 492c normally found in greater abundance as compared with isolate 492a were not reduced in abundance in the resistant mutants (Fig. 1 and 3). Thus, the outer membrane of isolate 492c is altered by the addition of two proteins as compared with a very similar isolate obtained from the same sputum sample.

The existence of a hypersusceptible strain of *P. aeruginosa* in a patient undergoing antibiotic therapy is perplexing and suggests that, if the hypersusceptibility is expressed in vivo, the isolate was not exposed to any significant level of antibiotic (25). Further, the extreme relatedness of isolate 492c to isolate 492a suggests that isolate 492c was originally isolate 492a but was modified due to the incorporation of a plasmid or temperate phage, with a resultant transition in outer membrane protein composition (3, 17, 27, 34). The origin of the hypersusceptibility of isolate 492c is presently under investigation.

If hypersusceptibility is expressed in vivo this would be further support for our previous sug-

gestion (9) that no single reason may explain the emergence of mucoid *P. aeruginosa* in CF patients, but that the reasons may be complex and interrelated, involving the consequences of long-term antibiotic therapy and the selective advantage of alginate in blocking *Pseudomonas* receptors on alveolar macrophages (J. W. R. Govan and J. A. M. Fyfe, manuscript in preparation) and phagocytic digestion (28).

An appreciation of heterogeneity and the existence and incidence of hypersusceptible *P. aeruginosa* in CF patients is important not only in understanding the reasons for the emergence of the mucoid form after primary infection with a nonmucoid isolate but in the pathogenesis and optimum treatment of existing infection.

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