Affinity for glycoproteins of bacteria found in the respiratory tract in cystic fibrosis

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SYNOPSIS The attraction of bacteria commonly found in the respiratory tract in cystic fibrosis to glycoprotein at pH 7 has been studied. The effect of washing on the removal of bacteria from the glycoprotein has also been investigated, and the value of doing both washed and unwashed sputum cultures is discussed.

The relationship between pathogenicity and the bacteria present in a routine sputum culture has been studied by Shiota (1967), who found that true pathogenic bacteria were more abundant in a culture from washed sputum than in a routine culture. Studies in this laboratory have shown that it is possible to increase the yield of *Haemophilus influenzae*, *Streptococcus pneumoniae* and, to some extent, *Staphylococcus aureus*, by this method.

May and Delves (1965) have shown that the ampicillin concentrations in sputum are influenced by the purulence of specimens, and Siggers and Lawson (1968) have demonstrated this relationship with cloxacillin in one case of cystic fibrosis. Bacteria that are embedded in the mucus would be difficult to eradicate with those antibiotics because of poor mucus penetration (Siggers and Lawson, 1966).

A series of experiments is reported here in which a bacteria-glycoprotein system *in vitro* has been used with a view to investigating the distribution of bacteria in mucus plugs and their attachment thereto.

Materials and Methods

TEST BACTERIA

These were all fresh isolates from routine cultures of cystic fibrosis sputum and were as follows:

a non-capsulated strain of *H. influenzae*, single strains of *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, a coagulase-positive staphylococcus, and *Strep. pneumoniae*.

All the above bacteria were used from overnight nutrient broth cultures grown at 37°C. X and V factors were added to the broth to support the growth of *H. influenzae*, and 1% horse blood was added to support the growth of *Strep. pneumoniae*.

BACTERIAL COUNTS

All dilutions of bacteria and bacteria plus glycoprotein were done in sterile Ringer's solution. The bacteria contained in 0.1 ml volumes of the dilutions were plated in duplicate by the spread plate method. Counts for *K. aerogenes*, *Ps. aeruginosa*, and *Staph. aureus* were done on nutrient agar, *H. influenzae* on X and V agar, *Proteus mirabilis* on McConkey agar, and *Strep. pneumoniae* on horse blood agar.

Plates showing less than 400 colonies were not counted. The mean bacterial count was determined from both sets of dilutions.

GLYCOPROTEIN

The design of the experiments required sterile glycoprotein of a fixed composition. The salivary glycoprotein from the same individual was used in all experiments to meet this requirement.

Mixed saliva was collected into sterile centrifuge tubes cooled in melting ice. The saliva was

centrifuged at 1,000 rpm for five minutes to remove any debris and the glycoprotein was precipitated at pH 3.0-4.0 with N acetic acid. The glycoprotein was redissolved in sterile Ringer's solution and refractionated with ethyl alcohol at 4°C at a final concentration of 64% (v/v). The use of ethyl alcohol in the procedure was sufficient to sterilize the glycoprotein. The refractionated glycoprotein was rehydrated in sterile Ringer's solution overnight at 4°C and then used.

All experiments in this series were done at pH 7.0. May and Delves (1964) have shown that the pH of the majority of fresh samples of bronchitic sputum are around this value, and in a limited study of cystic fibrosis sputum we have had similar results.

BACTERIAL CHARGE

The charge on the test bacteria was investigated at pH 7.0 using low voltage electrophoresis. A glass slide, 7.5 cm × 4.5 cm, was covered with 8 ml of 1% ion agar (Oxoid) in 0.1M phosphate/citrate buffer. A ditch, 60 mm × 3 mm, was cut into the agar and was filled with a suspension of the washed test organism at 1×10^2 organism/ml in Ringer's solution. Electrophoresis was run at 6 volts/cm for two hours, after which 2 mm loop samples were taken from the anodic and cathodic ends of the ditch and plated. The charge of the test bacterium was determined by migration in the electrical field.

The general charge on the glycoprotein molecule at pH 7.0 was determined by electrophoresis on cellulose acetate. The glycoprotein was stained by the periodic-acid Schiff method. Endosmotic flow in these experiments was determined by the migration of 2:4 dinitroethanolamine.

ATTACHMENT OF THE BACTERIA TO THE GLYCOPROTEIN

Two methods were used: by nephelometry, estimating the bacteria lost from a suspension by attachment to the glycoprotein, and by viable counts of the bacteria attached to the glycoprotein.

By nephelometry

In this series of experiments density readings were taken by using an EEL nephelometer with an OR2 filter. The supplied standard was set at 100% transmission.

Twenty millilitres of a suspension of washed bacteria in Ringer's solution was adjusted to give 50% transmission on the nephelometer. The bacterial suspension was placed at 4°C for one hour to ensure that all the bacteria were in the lag phase. Two 9 ml volumes of the suspension were taken; to one was added 1 ml of glycoprotein (test) and to the other

1 ml of sterile small glass beads (control). The addition of the glass beads (1.5 mm) was made to simulate trapping of bacteria by mucus sinking in the bacterial suspension.

The bacterial suspensions were then placed at 4°C for four hours and then the top 5 ml each of the test and control were read on the nephelometer. The control was also diluted 1:1, 1:5, 1:10 with sterile Ringer's solution and read. A total viable count was also done on the control to enable a graph of the number of bacteria per nephelometer reading to be constructed for each organism. The number of organisms removed from the suspension by the glycoprotein was calculated from this.

Attempts to run this experiment at 37°C were unsuccessful due to uneven bacterial growth and the tendency for the glycoprotein mass to disintegrate.

By viable count

Twenty millilitres of a 1×10^{-2} dilution of an overnight broth culture was prepared and then stored at 4°C for one hour. Nine ml of the bacterial suspension was added to each of two sterile centrifuge tubes. To one tube was added 1 ml of glycoprotein (test) and to the other tube 1 ml of Ringer's solution (control). Both tubes were incubated for four hours at 37°C and then treated as follows.

One millilitre was taken and an equal volume of buffered pancreatin solution (Oxoid) added. The mixture was incubated at 37°C for 30 min and counted. The results from this were taken as the basic count.

One millilitre of the control was counted without treatment with pancreatin, the remaining 7 ml was then spun for three min at 500 rpm and a 1 ml sample was taken from the top of the suspension and counted. The percentage of bacteria spun down was estimated and used as a correction factor for the test.

The bacterial suspension was removed from above the glycoprotein with a Pasteur pipette and the glycoprotein was washed once in 3 ml Ringer's solution. The resulting suspension was spun for three min at 500 rpm and the supernatant was removed. The glycoprotein plug was homogenized using an Atomix and divided into two 0.5 ml portions. To one portion was added 0.5 ml of buffered pancreatin, the mixture was incubated at 37°C for 30 min, and counted. The second portion was washed violently with three consecutive 3 ml volumes of Ringer's solution and then treated as the first portion.

Results

BACTERIAL CHARGE

The overall charges on the test bacteria were found to be: *H. influenzae* and *Strep. pneu-*

moniae, strongly negative; *Staph. aureus*, negative; *K. aerogenes*, *P. mirabilis*, and *Ps. aeruginosa*, weakly negative to neutral.

The total charge of the glycoprotein molecule was neutral to weakly positive.

ATTACHMENT OF BACTERIA TO GLYCOPROTEIN

The results of the two series of experiments (Tables I and II) have been calculated to give the number of bacteria from a suspension of 1×10^6 organisms that were attached to 1 ml of glycoprotein. Table I (nephelometry) shows that *H. influenzae*, *Strep. pneumoniae*, and *Staph. aureus* are more firmly attached to the glycoprotein than the other test organisms.

Organisms Supplied at 1×10^6 Organisms/ml	Mean No. of Organisms per ml of Glycoprotein
<i>Strep. pneumoniae</i>	8.0×10^5
<i>H. influenzae</i>	6.45×10^5
<i>Staph. aureus</i>	4.18×10^4
<i>Proteus mirabilis</i>	5.0×10^3
<i>Ps. aeruginosa</i>	1.4×10^3
<i>K. aerogenes</i>	7.3×10^2

Table I Attachment of bacteria to glycoprotein at pH 7.0 and a temperature of 4°C (by nephelometry)

Organism Supplied at 1×10^6 Organisms/ml	Mean No. of Organisms per ml of Glycoprotein	
	Before Washing	After Washing
<i>H. influenzae</i>	6.45×10^4	5.4×10^4
<i>Strep. pneumoniae</i>	6.25×10^4	6.2×10^4
<i>Staph. aureus</i>	5.7×10^3	4.3×10^3
<i>Proteus mirabilis</i>	4.1×10^3	3.7×10^3
<i>K. aerogenes</i>	1.55×10^3	1.1×10^3
<i>Ps. aeruginosa</i>	0	0

Table II Attachment of bacteria to glycoprotein at pH 7.0 and a temperature of 37°C (by viable count)

The results from the viable counts (Table II) show in general results similar to those obtained by nephelometry, with the strongly negatively charged bacteria again being more attracted to, or attached to, the glycoprotein.

In all cases washing the glycoprotein only slightly reduced the bacterial count.

Discussion

METHODOLOGY

The problem of the attachment of bacteria to glycoprotein has been studied by two methods, one measuring the bacteria remaining in suspension around the mucus and the other directly measuring the viable bacteria attached to the

mucus. In the design of the experiments correction has been made for some possible errors; it is worthwhile drawing attention to these so that the value of the results can be assessed.

In the nephelometric method errors due to the natural sedimentation of the bacteria were avoided by taking counts from the top of the suspension and by the addition of glass beads to the control to allow for bacteria trapped by the glycoprotein.

The necessity of running these experiments at 4°C might have had some effect on the degree of ionisation of the bacteria and the glycoprotein but this would only be slight as ionic suppression on the two system components would be similar. The normal errors also apply of measuring a suspension of viable and non-viable cells and estimating this as a total viable count. Estimating the bacteria by total N_2 was not feasible, as for the purpose of these experiments viable counts were required to give a representative picture of the attraction of bacteria to mucus. The method using direct viable counts to measure the bacteria on the glycoprotein had one drawback because centrifugation was essential to ensure that all the glycoprotein was estimated. Attempts were made to correct for spin down error in this series. As fragmentation of the glycoprotein did not matter, these experiments were run at 37°C.

Although the two methods suffered from different sources of error, the general patterns of the results were similar.

RESULTS

The bacteria that are normally considered pathogenic in the respiratory tract in cystic fibrosis, namely, *H. influenzae*, *Staph. aureus*, and *Strep. pneumoniae*, had a greater affinity for the glycoprotein than the other test organisms. This may be explained by the differing electrical charges on the bacteria. The removal of the bacteria from the glycoprotein by washing was very difficult and it is reasonable to assume that if a sputum specimen were treated in this way the loss of organisms from mucus plugs would be small. The effect of washing sputum would be to remove the debris which would contain a greater number of 'non-pathogens' than true pathogens. On culture of the washed sputum an increase in the proportion of pathogens would be expected.

The consequences of the differential attachment of bacteria to mucus may well be of importance in treatment, especially considered together with the differences in the degree of binding of various antibiotics to hog gastric mucus demonstrated by Siggers and Lawson (1966).

Work on this aspect in a glycoprotein-antibiotic and pus cell antibiotic system is at the moment being done in this laboratory and will be reported in due course.

It should also be borne in mind that specific mucolytic agents that attack glycoprotein would release pathogenic organisms from the glycoprotein, thus making them more susceptible to attack from antibiotics. It should not be forgotten, however, that in the absence of effective antibiotic therapy, mucolytics may increase the spread of the infection.

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