Proteus vulgaris agglutination by cystic fibrosis sera

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SUMMARY The factor in sera of patients with cystic fibrosis (CF) and their parents which agglutinates Proteus vulgaris has characteristics similar to those of IgG antibody to this organism. Sera of patients without CF who have P. vulgaris infections agglutinate the organism similarly. At present there are too many false-positives in a control population for the test to be widely useful for heterozygote identification.

Sera of patients with cystic fibrosis (CF) and their parents contain a factor which inhibits ciliary motility in a variety of assay systems including rabbit tracheal explants,1 oyster,2 or water mussel gills.3 Cohen and Daniel4 reported that suspensions of the flagellate bacterium Proteus vulgaris were agglutinated by such sera.

We have investigated the mechanism and specificity of the P. vulgaris agglutinating assay and its application as a screening test for CF gene heterozygotes in genetic counselling.

Materials and methods

Sera were obtained from 50 CF patients, from 57 of their parents (obligate heterozygotes), from 51 healthy adults (medical and other staff of the hospital), from 6 children infected by proteus (urinary, respiratory, or septicemic infections), from 3 of their parents, from 12 children with asthma, and from 38 children in hospital for various reasons. We also studied unstimulated whole mouth saliva of 23 CF patients and 25 of their parents, and nasal secretions from 2, urine from 8, and small intestinal juice from 11. The specimens were either tested immediately after collection, or stored at −20°C.

The P. vulgaris strain used was cultured from a child in the hospital, and was maintained at room temperature on nutrient agar. Before assay it was cultured in 20 ml nutrient broth at 37°C for 5 hours when an actively motile log phase was attained. One drop of bacterial suspension and one drop of the serum were dispensed by Pasteur pipette on to a microscope slide, covered with a coverslip, and examined for agglutination at × 400 magnification for 2-minute intervals for 25 minutes when the result was recorded as large agglutinates (>80 μm), small agglutinates (<80 μm), or no agglutination. We also used suspensions of Proteus mirabilis, Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae.

The effects of a variety of treatments on the agglutination of CF sera were studied: these were repeated freezing and thawing, heating at 56°C and 70°C for one hour, heparin, incubation in acid medium (pH 4·5 or 2·35), and precipitation of immunoglobulins with anti-immunoglobulin antisera (anti-IgM and anti-IgG, Wellcome Laboratories). The bacteria agglutinated by patients’ sera were studied by indirect immunofluorescence, using fluorescence-labelled sheep antihuman IgM and IgG (Wellcome Laboratories) at 1/10 dilution. Patients’ sera were separated on a Sephadex G200 column equilibrated with Tris-HCl buffer pH 7·6. IgG was prepared from patients’ sera by DEAE cellulose batch chromatography6 using Whatman DE-52 cellulose.

Ciliary motility tests were done with the gills of the fresh water mussel dreissensia.9 Gill segments were cut with scissors from freshly collected animals, and placed in closed moist plastic dishes containing 0·05 ml plasma and 0·10 ml distilled water; motility was observed intermittently for 6 hours using phase contrast illumination (× 300). Inhibition of mobility at 3 hours was classified as + +, inhibition between 3 and 6 hours as +, and no inhibition at 6 hours as negative.

Results

Most CF sera agglutinated P. vulgaris suspensions

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within 3 or 4 minutes. Sera from obligate CF heterozygotes agglutinated them rather more slowly and most sera from healthy adults had not agglutinated them by 25 minutes. The agglutinate formed by most CF sera were large (>80 μm in diameter), those formed by the sera from the CF parents were smaller—normally 10 to 40 μm in diameter. In a blind study, using this classification, a significant difference between the three groups of sera was obtained (Table 1). CF patients differed significantly from controls whether analysed for production of large agglutinates or agglutinates of any size (χ²>30, P<0.0005 for both); obligate heterozygotes differed from controls for agglutinates of any size (χ²=19.3, P<0.0005).

38% of healthy adults produced agglutinates, including large ones in 14%. Only 5% of such individuals would be expected to be heterozygous for the CF gene, so there are clearly mechanisms other than the CF gene leading to agglutination.

As reported by Cohen and Daniel the agglutinates withstood repeated freezing, thawing, and heating (at 56°C and 70°C) for one hour. Treatment of the serum with heparin did not prevent agglutination. The agglutinates were partially destroyed at pH 2.35 but not at pH 4.5.

The agglutinating factor in CF sera was not precipitated by dialysis against distilled water and so was shown to be a pseudoglobulin. After Sephadex G200 gel filtration of the CF sera, agglutination was detected in the middle (IgG-rich) peak in all patients but, in some, weak activity was also found in the first (IgM-rich) peak. IgG was prepared from the serum of a CF child by DEAE cellulose exchange chromatography; the activity remained with the IgG peak. Immunofluorescence examination showed that the agglutinates of P. vulgaris produced by CF sera contained IgG and occasionally IgM. Immunoprecipitation with anti-IgG antibody greatly reduced the agglutination and anti-IgM partly reduced it, but anti-IgA had no effect.

Sera from 3 CF patients, 2 CF mothers, and 2 healthy adults were studied for agglutination of 5 other flagellate bacteria. The same sera agglutinated P. mirabilis, though less strongly than the P. vulgaris (Table 2). The agglutination of the other bacteria did not closely parallel that of P. vulgaris.

These findings suggest that the factor is an agglutinating antibody to P. vulgaris, an organism which sometimes infects CF patients, and to which their parents may well have excessive contact and perhaps special vulnerability. This view is supported by the observation that the sera of the youngest patient (aged 3 months) and those of both his parents failed to agglutinate this organism.

Sera from 5 out of 6 children with proteus infection agglutinated P. vulgaris quickly into large agglutinates, as did the serum from one mother; some of the children with asthma and miscellaneous diseases did so too (Table 3). Sephadex and DEAE separation was consistent with this being IgG and IgM antibody. The urine of 8 CF patients positive for serum agglutination did not agglutinate P. vulgaris either neat or after 50-fold concentration by ultrafiltration. Saliva from 13 of 23 CF patients agglutinated P. vulgaris, 6 of them with large agglutinates, but only 6 of 18 of their parents, and only one of 10 healthy adults gave small agglutinates only (Table 4). In general, individuals gave both saliva and serum activity, but the saliva of one CF mother whose serum was negative did agglutinate P. vulgaris. Three out of the 11 small intestinal juice samples from CF children agglutinated P. vulgaris.

The relationship between the dreissensia gill cilia immobilisation test and P. vulgaris agglutination in

### Table 2 Agglutination of 5 bacterial species by sera from 3 CF patients, 2 CF heterozygotes, and 2 healthy subjects

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>CF Patients (n = 30)</th>
<th>CF Heterozygotes (n = 37)</th>
<th>Healthy Adult Controls (n = 51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteus mirabilis</td>
<td>++ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>++ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>+ + + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>+ + + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

### Table 3 P. vulgaris agglutination by patients with diseases other than CF

<table>
<thead>
<tr>
<th>Condition</th>
<th>Hospital Children (n = 28)</th>
<th>Asthma (n = 18)</th>
<th>Proteus Infection (n = 6)</th>
<th>Parent of Proteus Infected Children (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large agglutinates</td>
<td>6</td>
<td>3</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Small agglutinates</td>
<td>17</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No agglutination</td>
<td>15</td>
<td>7</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 4  Agglutination of P. vulgaris by saliva of CF homozygotes and heterozygotes

<table>
<thead>
<tr>
<th></th>
<th>CF patients (n = 23)</th>
<th>CF parents (n = 23)</th>
<th>Healthy adults (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large agglutinates</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Small agglutinates</td>
<td>7</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>No agglutinates</td>
<td>10</td>
<td>17</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 5  Association between P. vulgaris agglutination and ciliary immobilisation test in sera of CF patients and their parents

<table>
<thead>
<tr>
<th>P. vulgaris agglutination</th>
<th>CF patients</th>
<th>CF parents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large agglutinates</td>
<td>+ + 5</td>
<td>+ + 1</td>
</tr>
<tr>
<td>Small agglutinates</td>
<td>+ + 0</td>
<td>+ + 1</td>
</tr>
<tr>
<td>No agglutinates</td>
<td>+ 0</td>
<td>+ 1</td>
</tr>
</tbody>
</table>

CF patients and their parents is shown in Table 5. Analysis of positive or negative reactions for the two tests on children and parents combined shows a highly significant relationship (P = 0.004, Fisher's exact test). The one child without CF who had proteus infection and whose serum agglutinated P. vulgaris gave an intermediate value for ciliary immobilisation.

Discussion

These results confirm the report that sera from patients with CF and their parents agglutinate P. vulgaris more frequently, quickly, and strongly than do sera from healthy subjects.4 It is closely associated with the previously reported immobilisation of mammalian or invertebrate cilia.1-3 The observation that parents (obligate heterozygotes) gave intermediate values raised the possibility that the factor was closely related to the primary gene product but there are other hypotheses for it. Our results suggest that the factor concerned is an IgG antibody to P. vulgaris; the evidence included gel filtration and ion exchange chromatography separation, inhibition by anti-IgG antiserum, and immunofluorescence analyses of the agglutinates. Sera of patients with proteus infection similarly agglutinated P. vulgaris and the factor concerned had similar characteristics. Since few people become ill with proteus infection and 5% of the population are thought to be heterozygotes for CF,6 it is possible that these individuals were heterozygotes and had an increased susceptibility to proteus, but the frequency of agglutination in the healthy population was too high for this to be the only explanation, and it is possible that the test results from previous sensitisation to a proteus or an antigenically cross-reacting organism. Bowman et al.7 similarly failed to separate the ciliary immobilisation factor from IgG although they subsequently reported9 that CF fibroblast cultures released a protein which caused ciliary immobilisation and suggested that this might associate with IgG in vivo. This concept requires further study. Wilson et al.9-10 reported abnormal cationic protein bands after isoelectric focusing of sera from CF homozygotes and heterozygotes, but did not directly investigate whether these were IgG or not; their findings are therefore not incompatible with ours.

As CF patients are often infected with flagellate bacteria, including P. vulgaris, and it is likely that their parents are therefore overexposed to such a contact, the test may simply be detecting antibody to these organisms, occurring as a tertiary effect of the disease in both the patients and their parents, as close contacts rather than as a direct effect of the gene. It is also possible that the incidence we observed in our healthy population might be misleadingly high, since they, as hospital workers, may be abnormally exposed to such infections. Since bacterial flagellae and cilia of other organisms are structurally similar, it is also possible that the ciliary immobilisation tests result from the same sensitisation, if there is antigenic cross-reaction between these structures.

The close relationship between the P. vulgaris agglutinating test and the ciliary immobilisation test, and the negative results in the one CF infant studied and his parents supports this view, but the finding that agglutination of P. vulgaris was closely related to that of P. mirabilis but not to agglutination of other flagellates is to some extent against it. More work on such possible cross-reactions is needed.

Before these tests can have any value in identifying heterozygotes as family members at risk, for screening their potential spouses, or for epidemiological work (for example, the role of CF heterozygosity in atopy)11 we must know more about their mechanism. If the abnormal incidence of P. vulgaris agglutination occurs as a result of abnormal contact with flagellate bacteria it would be useless; if it is an abnormal response to a normal contact with an environmental factor, it could have some limited value as has been suggested for atopy,11 although many false-positives would be expected. It is only if it is closely related to the primary gene product that the tests could be of real use.

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