

## Serum antibodies to *Pseudomonas aeruginosa* in cystic fibrosis

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**SUMMARY** Serum IgG antibodies to *Pseudomonas aeruginosa* cell surface antigens were determined by enzyme linked immunosorbent assay. Titres in patients without cystic fibrosis were low (140–235). Those in patients with cystic fibrosis who were chronically infected by *P. aeruginosa* were very high (1100–20 500), while patients who grew the organism intermittently had lower titres (160–4400). Longitudinal studies showed that raised titres were observed at a very early stage of infection. High titres were associated with a poor clinical state, while low titres were associated with a better clinical state in both chronic and intermittently infected patients with cystic fibrosis. These results suggest that this test is a specific and sensitive measure of the severity and progress of the different stages of pulmonary infection by *P. aeruginosa* in patients with cystic fibrosis.

One of the most common causes of morbidity and subsequent mortality in cystic fibrosis is pulmonary infection, particularly by *Pseudomonas aeruginosa*.<sup>1</sup> Once *P. aeruginosa* is established in the lungs it is extremely difficult to eradicate, despite repeated courses of chemotherapy.

At present, there is no specific measure of the severity and progress of the early stages of *P. aeruginosa* infection. Growth of *P. aeruginosa* from the respiratory tract may be due to harmless colonisation or to an active infection.

The purpose of this study was to measure immunological changes associated with colonisation and infection of the respiratory tract by *P. aeruginosa* and to assess the value of these measurements in monitoring the progress of pulmonary infection.

### Patients and methods

**Patients.** Serum samples, surplus to requirements, were obtained from 75 patients with cystic fibrosis who attended the paediatric and adult cystic fibrosis clinics at our hospital during a study period of 12 months. Diagnosis of the disease was confirmed by at least one sweat test.<sup>2</sup> The patients were divided into three groups. Group 1 comprised 31 patients with cystic fibrosis (20 girls and 11 boys), who had

an age range of 1–17 years, with a mean (SE) of 15.4 (7.3) years. *P. aeruginosa* had never been isolated from their sputum. Group 2 comprised 22 patients with cystic fibrosis (12 girls and 10 boys), who had an age range of 4–25 years, with a mean (SE) of 11.8 (1.5) years. *P. aeruginosa* had been isolated from their sputum at least once, but they had no recognisable illness due to that organism. Group 3 comprised 22 patients with cystic fibrosis (11 girls and 11 boys), who had an age range of 6–23 years, with a mean (SE) of 15.7 (4.6) years. *P. aeruginosa* had been isolated continuously from their sputum for at least a year, and they had recognisable chest illness due to that organism. Two serum samples at least 12 months apart were available from five patients in group 2 and seven patients in group 3.

Overall clinical state was assessed by the Shwachman score.<sup>3</sup> A maximum of 25 points was awarded for each of the following: chest x ray film, nutrition, general activity, and physical examination. Declining scores indicated a worsening condition. Chest x ray films were assessed by the Chrispin-Norman score.<sup>4</sup> The possible scores were 0–38, with increasing score indicating increasing abnormality. Complete clinical evaluation within three days after the serum sample had been obtained, and repeated bacteriological results, were available for 16

patients. Serial serum samples were available for nine of these patients. All these patients are reported on.

#### Controls

The control group consisted of 17 patients (seven girls and 10 boys) who were attending the paediatric clinic at this hospital and from whom blood specimens were taken surplus to requirements. The mean (SE) age was 9.54 (1.42) years, ranging from 5 months to 15 years. None had any evidence of lung disease or *P. aeruginosa* infection. There were three cases of diabetes, two each of anaemia, short stature, severe retardation, and haematemesis, and one each of lethargy, intestinal polyp, oesophageal reflux, malaria, congenital emphysema, and hypothyroidism.

**Serum.** All samples were stored at  $-20^{\circ}\text{C}$  until immediately before use.

**Enzyme linked immunosorbent assay (ELISA).** IgG antibodies to *P. aeruginosa* cell surface antigens were measured by ELISA. Seven strains of *P. aeruginosa* with serotypes 1, 3, 6, 9, 10, 11, and a strain that could not be serotyped were used.<sup>5</sup> These are the most commonly isolated serotypes in our unit. Washed cells were bound to microtitre plates (Immulon Grade A, Dynatech) then fixed by 0.5% vol/vol glutaraldehyde in phosphate buffered saline. Unbound sites were blocked by 1% vol/vol bovine serum albumin in phosphate buffered saline. A single serotype was used in each plate.

A pool of serum with high titres, diluted between 1:1500 and 1:50 000 in 1% bovine serum albumin in phosphate buffered saline, was used for a standard curve on each plate. One hundred microlitres of each standard and test serum was added and incubated in triplicate for 75 minutes at room temperature. After washing, 100  $\mu\text{l}$  goat antihuman IgG ( $\gamma$  chain specific) conjugated to horseradish peroxidase (Zymed Laboratories, San Francisco) diluted 1:2000 was added and then incubated for two hours at room temperature. After washing, 100  $\mu\text{l}$  enzyme substrate solution, containing 5 mg orthophenyldiamine and 20  $\mu\text{l}$  of 12% vol/vol hydrogen peroxide in 10 ml citrate phosphate buffer, was added. After two minutes the reaction was stopped by the addition of 100  $\mu\text{l}$  4M sulphuric acid. Absorption at 492 nm was measured in a Titerack plate reader. Absorbance was  $\leq 0.10$  in wells not containing any one of the following alone: adsorbed antigen; test serum; or labelled antibody.

The blank absorbance was subtracted from the test absorbance. This value was converted to a dilution by reference to the standard curve. The

reciprocal of the dilution used for the patient's serum (1000 or 10 000) was divided by the reciprocal of the dilution obtained from the standard curve, multiplied by 1000 and expressed as a titre. Each serum sample was tested against all seven antigens on the same day. The titre presented here is the sum of these seven values.

**Statistical analysis.** The Student's *t* test, regression analysis to estimate the best fit, and Kendall's rank correlation test were used. Significance levels were for two tailed tests.<sup>6</sup>

#### Results

The serum IgG antibody titre against *P. aeruginosa* from the 17 control patients is shown in Figure 1. Titres ranged from 140–250. The mean (SE) was 200 (5.6). The upper limit (99.9% confidence limits) was 270. There was no correlation between titre and either age or sex.

In each of the three groups of patients there was a wide spread of IgG titres against *P. aeruginosa* (Fig. 1). The highest titres were in patients who grew *P. aeruginosa* continuously (group 3). Patients from whom this organism had been isolated intermittently (group 2) generally had lower titres, although there was some overlap between the two groups. There was a significant difference between the titres in these two groups ( $p < 0.002$ ). Titres of patients in group 1 (*P. aeruginosa* never isolated from sputum) varied between 140 and 805 and were significantly lower than group 2 ( $p < 0.1$ ) and group 3 ( $p < 0.001$ ). Titres of all three groups of patients with cystic fibrosis were significantly higher than the control group ( $p < 0.001$  for group 3,  $p < 0.05$  for group 2, and  $p < 0.1$  for group 1).

Titres from patients in group 2 fell into three subgroups. Titres of most patients (14/22) were between 300 and 700. Four patients, however, had titres greater than 1000, which were within the range of patients in group 3. Three of these four patients were tested twice, with an interval of one year between samples. *P. aeruginosa* had been isolated on five previous monthly investigations from one of these patients and continuously for four months from another patient. These patients may be on the borderline between group 2 and group 3. Another patient had a poor record of attendance at clinic and had not been examined in the previous 12 months, while *P. aeruginosa* had been isolated intermittently for nearly five years from the fourth patient. At the other extreme, there were four patients also in group 2 who had titres of not more than 200, well within the control range. All these serum samples

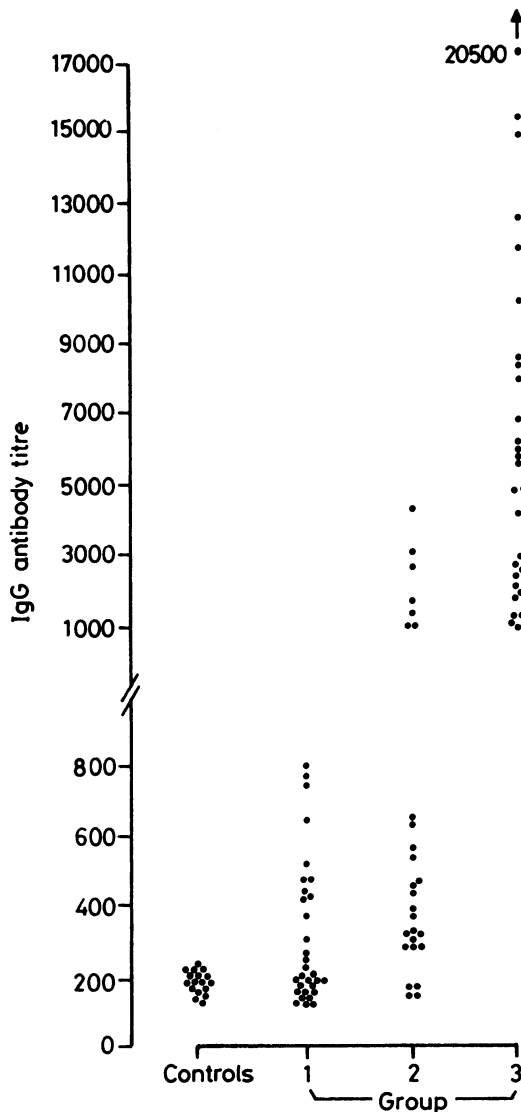


Fig. 1 Serum IgG antibody titre to *P. aeruginosa* in patients with and without cystic fibrosis. IgG antibody titre is to *P. aeruginosa* serotypes 1, 3, 6, 9, 10, 11 and a strain that could not be serotyped.

Controls: paediatric patients with no history of *P. aeruginosa* infection; group 1: patients with cystic fibrosis from whom *P. aeruginosa* had never been isolated; group 2: patients with cystic fibrosis from whom *P. aeruginosa* had been isolated at least once; group 3: patients with cystic fibrosis from whom *P. aeruginosa* had been isolated continuously for at least one year.

were obtained eight to 12 months after a single isolation of *P. aeruginosa* from sputum.

Titres from patients in group 1 fell into two subgroups. Nineteen had titres less than 300, which were within the normal range, and 12 had titres greater than 300, which were greater than the normal range.

These results showed that there was considerable variation in the titre of IgG antibodies to *P. aeruginosa* surface antigens among patients with cystic fibrosis. High titres were associated with patients in group 3. There was some overlap between titres found in patients in group 3 (chronic *P. aeruginosa* isolation) and patients in group 2 (intermittent *P. aeruginosa* isolation). Conversely, there were four patients in group 2 who had titres within the normal range.

Having established that there were differences in IgG titre to *P. aeruginosa* surface antigens among patients with cystic fibrosis, we evaluated the clinical importance of these findings. Clinical data, obtained within three days of the serum collection, was available for seven patients in group 2 and nine patients in group 3. It was thought that patients in group 1 deserved a separate study and are reported on elsewhere. The relation between IgG titre and Shwachman score is shown in Figure 2 and between IgG titre and Chrispin-Norman chest x ray score in Figure 3. There was a strong correlation between increasing IgG titre and deteriorating clinical state as measured by the Shwachman score ( $\tau = -0.6385$ ,  $p < 0.01$ ). There was also a correlation between IgG titre and lung damage as measured by Chrispin-Norman x ray score ( $\tau = +0.4417$ ,  $p < 0.10$ ).

Two or more serum samples were available from seven patients in group 3. They all behaved in a similar manner. Representative results of longitudinal studies for two patients in group 3 are shown in Figure 4. The IgG titre increased in all patients we investigated, even in those given repeated courses of intravenous treatment with antibiotics. The rate of

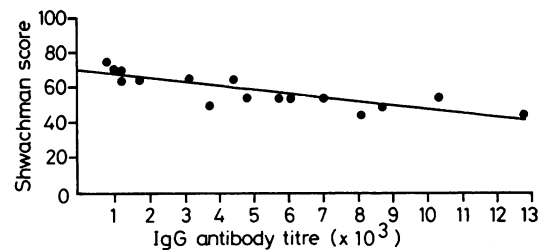


Fig. 2 Relation between Shwachman score and serum IgG antibody titre to *P. aeruginosa* in patients with cystic fibrosis.

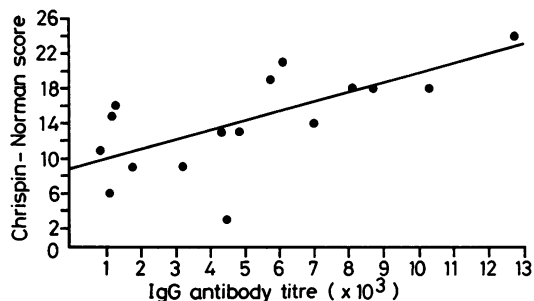


Fig. 3 Relation between Chrispin-Norman score and serum IgG antibody titre in patients with cystic fibrosis.

increase in titre varied in different patients. A rapid increase in titre was accompanied by a rapid decline in Shwachman score. Conversely, a slow increase in titre was accompanied by a more stable clinical state.

Longitudinal studies in two patients who were on the borderline between groups 2 and 3 are shown in

Figure 5. In one patient *P. aeruginosa* had been present continuously since the initial isolation from sputum. In the other, *P. aeruginosa* had been isolated intermittently for three years. Intravenous anti-pseudomonal treatment was followed by a decrease in titre, after a lag of six to 10 weeks, in both patients. Throughout the period of investigation, changes in titre were the opposite of changes in Shwachman score in these patients.

Discussion

The purpose of this study was to assess the value of the measurement, by ELISA, of IgG antibodies to *P. aeruginosa* in monitoring the progress of pulmonary infection by the organism in patients with cystic fibrosis. Serum was tested against seven strains of *P. aeruginosa* with serotypes 1, 3, 6, 9, 10, 11 and a strain that could not be serotyped.<sup>5</sup> These represented 85% of isolates obtained from our patients during the previous six months.

Antibodies reacting with *P. aeruginosa* were

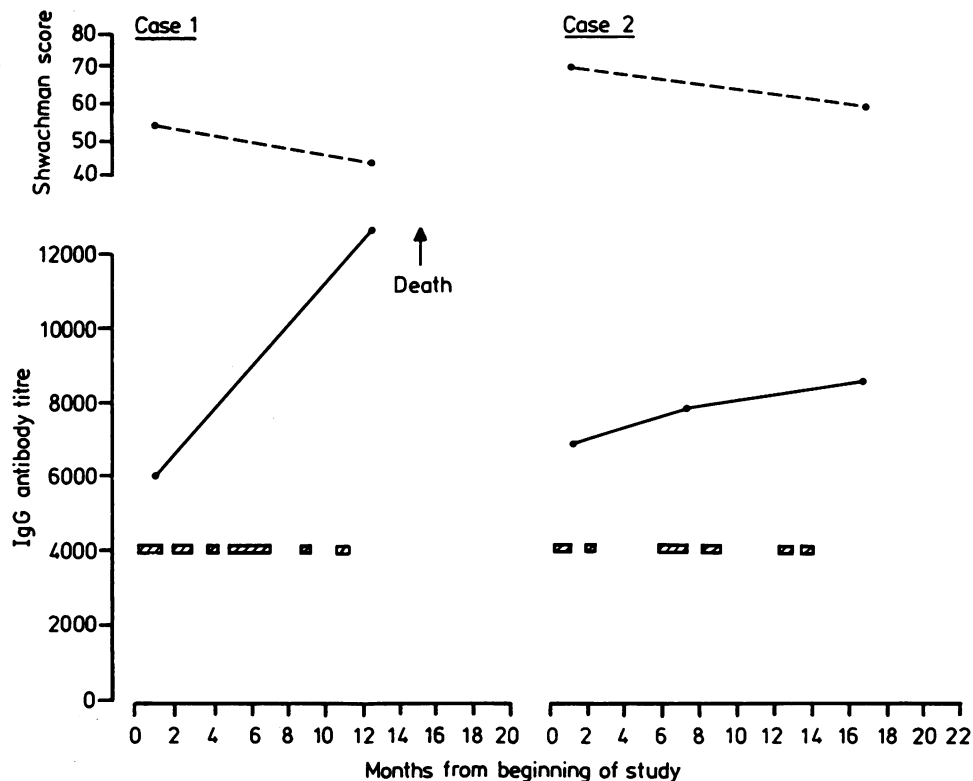


Fig. 4 Longitudinal studies of serum IgG antibody titre and Shwachman score in two patients with cystic fibrosis chronically infected by *P. aeruginosa* (group 3). Hatched areas represent intravenous treatment with antibiotics.

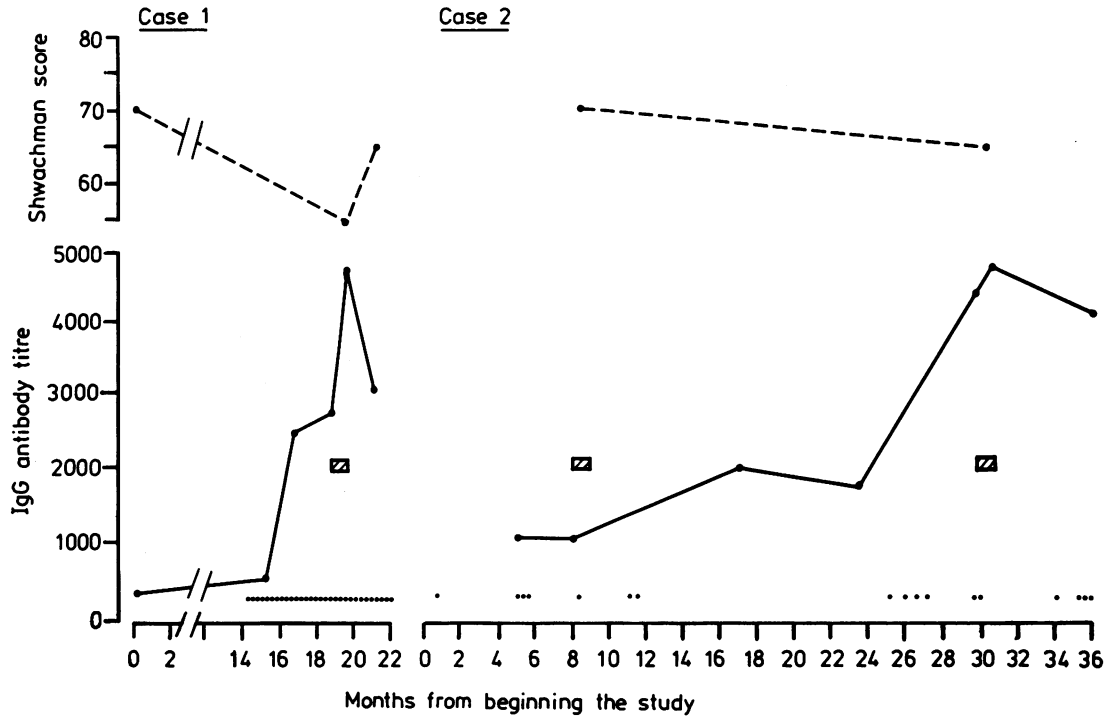


Fig. 5 Longitudinal studies of serum IgG antibody titre and Shwachman score in two patients with cystic fibrosis in group 2. Hatched areas represent intravenous treatment with antibiotics. Dots denote *P. aeruginosa* isolated from sputum.

present at low concentrations in patients without cystic fibrosis with no known *P. aeruginosa* infection. The mean titre was 200 and the upper limit (99.9% confidence limits) was 270. Hoiby, using counterimmunoelectrophoresis, has shown that the prevalence of precipitating antibodies against sonicated *P. aeruginosa* increased gradually with age in a normal population. These antibodies were present only at low titres, however, and were directed against two antigens that crossreacted with antigens from other bacterial species.<sup>7,8</sup> In a survey of 100 adults of unspecified age, using ELISA, serum antibodies against *P. aeruginosa* surface antigens were also present at low concentrations.<sup>9</sup> One of our previous studies showed that crossreacting antibodies primarily directed against other Gram negative organisms did not make a significant contribution to the titre against *P. aeruginosa* in this assay.<sup>10</sup> Any titre greater than 300 was therefore considered greater than normal and indicative of the presence of a specific humoral response to *P. aeruginosa*.

Patients with cystic fibrosis who grew *P. aeruginosa* continuously had very high IgG titres, while patients who grew the organism intermittently

generally had lower titres. There was some overlap, however, between the two groups of patients. This presumably reflects the arbitrary division between the two groups. In contrast, other patients who grew *P. aeruginosa* intermittently had IgG titres within the range of normal values several months after a single isolation of *P. aeruginosa* from their sputum. The absence of a systemic humoral response may be due to the presence of *P. aeruginosa* in sputum that was transient or non-pathogenic. Alternatively, the absence of a systemic humoral response may be due to an early and adequate host response utilising secretory IgA antibodies or to an inadequate systemic immune response to the infection. It is unlikely that antibodies to *P. aeruginosa* were present in these patients' serum but not detected by this test. The serotypes of *P. aeruginosa* isolates from all these patients were among those included in the assay. In addition, the outer membrane proteins of *P. aeruginosa* are highly conserved.<sup>11,12</sup> As used in this test they are likely to be accessible to antibodies present in the serum and should give a positive result. The possibility of an inadequate systemic immune response is unlikely. The serum IgG concentrations in

these patients were 10.1 g/l, 13.1 g/l, 13.7 g/l, and 13.8 g/l, which are within the normal range (5–16 g/l) for their ages. Work is in progress to examine these possibilities.

High IgG titres were associated with poor clinical state, as measured by Shwachman score, and with evidence of lung damage, as measured by Chrispin-Norman x ray scores. Conversely, a low titre was associated with a good clinical state and x ray score. This was true for patients who grew *P. aeruginosa* continuously or intermittently.

These results confirm and extend those previously reported by Hoiby.<sup>13–15</sup> Using a sonicated preparation of *P. aeruginosa* cells and counterimmunoelectrophoresis, the number of precipitating antibodies increased with increasing duration of infection and correlated with a decrease in clinical state, but only in patients who had grown *P. aeruginosa* continuously for at least six months. The method used in this study showed an increase in IgG titres above control values at an earlier stage of infection. There are three possible reasons for this. First, ELISA is much more sensitive than counterimmunoelectrophoresis. Secondly, the antigen used was different. The antigen used in this work consisted of whole glutaraldehyde treated cells—that is, cell surface antigens that are in their native configuration. These are likely to be the major antigenic stimulus in the earliest stages of infection. Finally, only precipitating antibodies are detected by counterimmunoelectrophoresis, while both precipitating and non-precipitating antibodies are detected by ELISA.

Longitudinal studies revealed that the rate of increase in titre was fast or slow in different patients. The titres did not decrease in the seven patients studied who had grown *P. aeruginosa* for several years and who had high titres, even if they had received repeated courses of intravenous treatment with antibiotics. The continued antibody presence implies the continued presence of the appropriate antigen. These findings are in agreement with those of Doring and Hoiby.<sup>16</sup> A decrease in titres was observed, however, after intravenous treatment with antibiotics in patients who had grown *P. aeruginosa* for shorter periods. This decrease was apparent some weeks or months after treatment, in accordance with the normal delay in response by the humoral immune system.

Twelve of 31 patients with no known *P. aeruginosa* infection had titres greater than the normal range. Only one patient had an infection with any Gram negative organism. Antibodies directed against Gram negative organisms other than *P. aeruginosa* do not make a significant contribution to this assay.<sup>10</sup> It is extremely unlikely, therefore, that

crossreacting antibodies were the cause of these raised titres but rather that they were indicative of a specific humoral response to *P. aeruginosa*. Most of these patients were fairly well and produced little or no sputum. Bacteriological findings were therefore based on throat swabs. Even in those patients who did produce sputum, organisms present in low numbers (less than 10<sup>4</sup> per ml) are unlikely to be detected by the protocol used in our laboratory. Longitudinal examination of the bacteriology and IgG titres of these patients is continuing.

The measurement of free serum IgG antibodies to *P. aeruginosa* by ELISA is now possible on a routine basis. It provides a specific, quantitative, and highly sensitive measure of immunological changes associated with all stages of pulmonary infection by *P. aeruginosa*. These changes correlate with changes in both the clinical state and the chest x ray film. The results presented here suggest that the ELISA may be a suitable test to differentiate between colonisation by *P. aeruginosa* (presence of the organism without appreciable tissue damage) and infection by *P. aeruginosa* (tissue invasion and damage). Longitudinal studies are in progress to provide further proof of this. Of particular importance is the ability to detect a systemic immune response to *P. aeruginosa*, and hence putative tissue invasion, before it is isolated from sputum. This may have far reaching implications on the treatment and prognosis of patients.

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