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Serum levels and *in vitro* production of IgA subclasses in patients with primary IgA nephropathy

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SUMMARY

Patients with primary IgA nephropathy have deposits of IgA1 in their kidneys, and increased plasma levels of macromolecular IgA1. Total serum IgA concentrations are frequently elevated, but studies on the subclass distribution have been few and conflicting. Several investigators found that production of IgA by peripheral blood lymphocytes in culture is increased. However, the distribution of the IgA subclasses produced has not been studied previously. We studied the serum IgA subclasses in 14 patients with IgA nephropathy, and found a significant ($P < 0.001$) increase in IgA1 (3.71 ± 1.34 mg/ml, mean $\pm$ s.d.) compared with controls (1.77 ± 1.10 mg/ml). Serum IgA2 was not different in patients and controls. The ratio of serum IgA1 to total IgA was also significantly ($P < 0.001$) higher in patients ($92.2 \pm 4.9\%$) than in controls ($80.2 \pm 6.6\%$).

Studies of immunoglobulin production by peripheral blood mononuclear cells showed a significant increase in IgA1 synthesis, expressed as a fraction of total IgA synthesis in unstimulated cultures ($P < 0.05$) and in PWM stimulated cultures ($P < 0.01$). Polymeric IgA and polymeric IgA1 production were not higher in patients than in controls. IgM production in unstimulated cultures was significantly ($P < 0.05$) higher in patients than in controls. Together with the observed deposition of exclusively IgA1 in the mesangium, our results indicate that patients with IgA nephropathy preferentially produce antibodies of the IgA1 subclass.

Keywords IgA nephropathy IgA subclasses immunoglobulin production polymeric IgA

INTRODUCTION

Primary IgA nephropathy is characterized by the deposition of IgA in the glomerular mesangium. Most investigators now agree that the IgA deposited in the glomeruli is of the subclass IgA1 (Conley, Cooper & Michael 1980; Tomino *et al.*, 1981; Valentijn *et al.*, 1984; Rajamaran, Goldblum & Cavallo, 1986). An as yet undetermined percentage of the IgA in the deposits is in a polymeric form (Béné *et al.*, 1982; Tomino *et al.*, 1982; Lomax-Smith *et al.*, 1983; Valentijn *et al.*, 1984). Numerous studies point to an abnormality of immune regulation in patients with IgA nephropathy, since an increased production of IgA by peripheral blood mononuclear cells *in vitro* was observed (Egido *et al.*, 1982; Bannister *et al.*, 1983; Egido *et al.*, 1983; Cagnoli *et al.*, 1985; Casanueva *et al.*, 1986; Feehally *et al.*, 1986; Hale *et al.*, 1986; Schena *et al.*, 1986; Waldo *et al.*, 1986). The IgA produced contains an increased fraction of polymers (Egido *et al.*, 1982; Lozano, Garcia-Hoyo & Egido, 1987). This increased production has been related to decreased IgA specific suppressor T cell activity (Sakai, Nomoto & Arimori 1979) and increased IgA specific helper Tα cells (Sakai *et al.*, 1982) in these patients.

In the circulation of patients with IgA nephropathy increased levels of polymeric IgA are found by some investigators (Lopez Trascasa *et al.*, 1980; Valentijn *et al.*, 1983), restricted to the subclass IgA1 (Valentijn *et al.*, 1984). Others found no increase in the ratio of polymeric over total serum IgA (Woodroffe *et al.*, 1980; Lesavre *et al.*, 1982; Delacroix *et al.*, 1983). Analysis of serum IgA subclasses in a small group of patients revealed a non-significant increase in the percentage of IgA1 (Delacroix *et al.*, 1983). Others failed to find abnormalities in the serum concentrations of the IgA subclasses, but have not studied the relative contributions of both subclasses to total IgA (Cosio *et al.*, 1982). Circulating immune complex-like material in these patients may not only consist of polymeric IgA1, but also of monomeric IgA1 complexed with IgG and C3 (Czerkinsky *et al.*, 1986).

To our knowledge no studies have been performed concerning the IgA subclass production in peripheral blood mono-

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nuclear cell culture. The present study was undertaken to investigate this aspect, and to correlate the findings with the serum levels of the IgA subclasses.

MATERIALS AND METHODS

Subjects

Fourteen patients with biopsy proven primary IgA nephropathy were studied (11 males and three females, mean age 32·4 years, range 18–44). The diagnostic criteria were presence of mesangial IgA, with or without C3 and other immunoglobulins, in the absence of clinical or laboratory evidence of systemic lupus erythematosus, liver disease, Henoch–Schoenlein purpura, or other systemic disease. The control group consisted of sixteen healthy hospital employees (14 males and two females, mean age 36·4 years, range 27–51). Neither patients nor controls had suffered mucosal infections in the two weeks preceding the study. None of the patients had macroscopic haematuria at the time of the study.

Cell culture and sera

Sera were obtained from fresh venous blood, and stored at −20°C. Peripheral blood mononuclear cell suspensions were prepared from heparinized fresh venous blood by standard Ficoll–Isopaque density gradient centrifugation. The cells were washed twice in Hanks' Balanced Salt Solution and once in complete culture medium, consisting of RPMI 1640 (Flow Laboratories, Zwanenburg, The Netherlands) supplemented with 10% heat inactivated fetal calf serum (Gibco Ltd, Paisley, Scotland), 2 mM L-glutamine, 12 mM $NaHCO_3$, 20 mM HEPES buffer (Sigma Chemical Company, St Louis Mo, USA), penicillin (100 U/l), and streptomycin (100 mg/l) (Sigma Chemical Company). After removing cell aggregates by cotton wool filtering, both unstimulated and pokeweed mitogen (PWM) stimulated (PWM 1:150 v/v; Gibco Ltd) cultures were performed. All cultures were performed in duplicate at 1×10^6/ml in round-bottom 12 × 75 mm polystyrene test tubes (Falcon 2058, Becton Dickinson and Co, Cockeysville, Md, USA) containing 1 ml of the cell suspension in a moist air atmosphere with 5% CO_2 at 37°C. At the end of the 7 day culture period the cells were resuspended, the test tubes centrifuged, and the cell free supernatants stored at −20°C until further use.

Immunoglobulin determination

Total serum IgA was determined by radial immunodiffusion. Culture supernatant immunoglobulins and serum IgA subclasses were measured by solid phase sandwich enzyme-linked immunosorbent assay (ELISA) as previously described (Van den Wall Bake *et al.*, 1988). Concentrations in test samples were calculated from standard curves obtained in each assay using dilutions of human plasma containing known concentrations of IgG, IgA, IgM, and IgA subclasses. Calculations were made at sample dilutions yielding an optical density in the linear part of the calibration curve. All determinations were performed in duplicate, and the final concentration expressed as the mean of duplicates for sera or cultures. Total concentrations of IgA1 and IgA2 were calculated by multiplying the measured ratio of the subclass over the sum of both subclasses with the measured total IgA concentration.

High performance liquid chromatography (HPLC)

Molecular sieving HPLC was performed on the PWM stimulated peripheral blood mononuclear cell culture supernatants using a TSK G3000 SW column connected via a guard column to an HPLC pump (LKB, Bromma, Sweden). Culture supernatants were passed through a 0·22 μM filter (Millipore Corp., Bedford, Mass.) and an undiluted 200 μl sample injected on to the column. The column was equilibrated and run in 0·1 M phosphate buffer with 0·1 M NaCl, pH 6·8, at a flow rate of 0·3 ml/min. One minute fractions were collected and assayed for total IgA content by ELISA. Based on the IgA profile, fractions were pooled to form a polymeric and a monomeric pool for each supernatant. The volumes of each pool were calculated by multiplying the number of fractions with the average fraction volume measured in each HPLC run. Contents of total IgA and IgA subclasses were determined in each polymeric and monomeric pool by measuring concentrations of IgA and IgA subclasses by ELISA, and multiplying these concentrations with the pool volume. The percentage of polymers in each supernatant was determined by dividing the polymeric content by the sum of polymeric and monomeric content.

Secretory component binding

Secretory component (SC) was isolated from human whey as previously described (Hiemstra *et al.*, 1988), and used as a reagent to detect polymeric IgA in the PWM stimulated peripheral blood mononuclear cell cultures. ELISA plates were coated with an IgA specific mouse monoclonal antibody (NI 184, Nordic Immunological Laboratories, Tilburg, The Netherlands) in carbonate buffer pH 9·6 for 2 h at 37°C. After three washes with phosphate-buffered saline containing 0·05% Tween 20 (PBS-Tween) culture supernatants, diluted in PBS-Tween containing 1% heated newborn calf serum were incubated for 2 hours at 37°C, followed by three washes with PBS-Tween. Secretory Component (SC) diluted to 12 μg/ml in PBS-Tween containing 0·5% bovine serum albumin (Sigma) was added. After 2 hours at room temperature incubation was continued overnight at 4°C. After washing, bound SC was detected with a biotin-coupled mouse monoclonal antibody to SC (NI 194, Nordic) followed by streptavidin conjugated to horseradish peroxidase (Zymed, Sanbio BV, Uden, The Netherlands) as described above. Dilutions of a human polymeric IgA standard, prepared using gel filtration, were included in each assay, and sample polymeric IgA concentrations were calculated from a standard curve.

Statistical analysis

Differences in immunoglobulin concentrations, ratios of immunoglobulins or percentages of polymeric immunoglobulins were assessed with a Mann–Whitney *U*-test. Results are expressed as means ± s.d.

RESULTS

Studies of serum IgA

The concentration of total IgA and both IgA subclasses is shown in Fig. 1. Patients have a significantly higher serum concentration of total IgA (4·01 ± 1·38 versus 2·13 ± 1·17 mg/ml, $P < 0·01$) and IgA1 (3·71 ± 1·34 versus 1·77 ± 1·10 mg/ml, $P < 0·001$) compared with normal controls. The ratio of IgA1 over total IgA is also significantly higher in patients than in

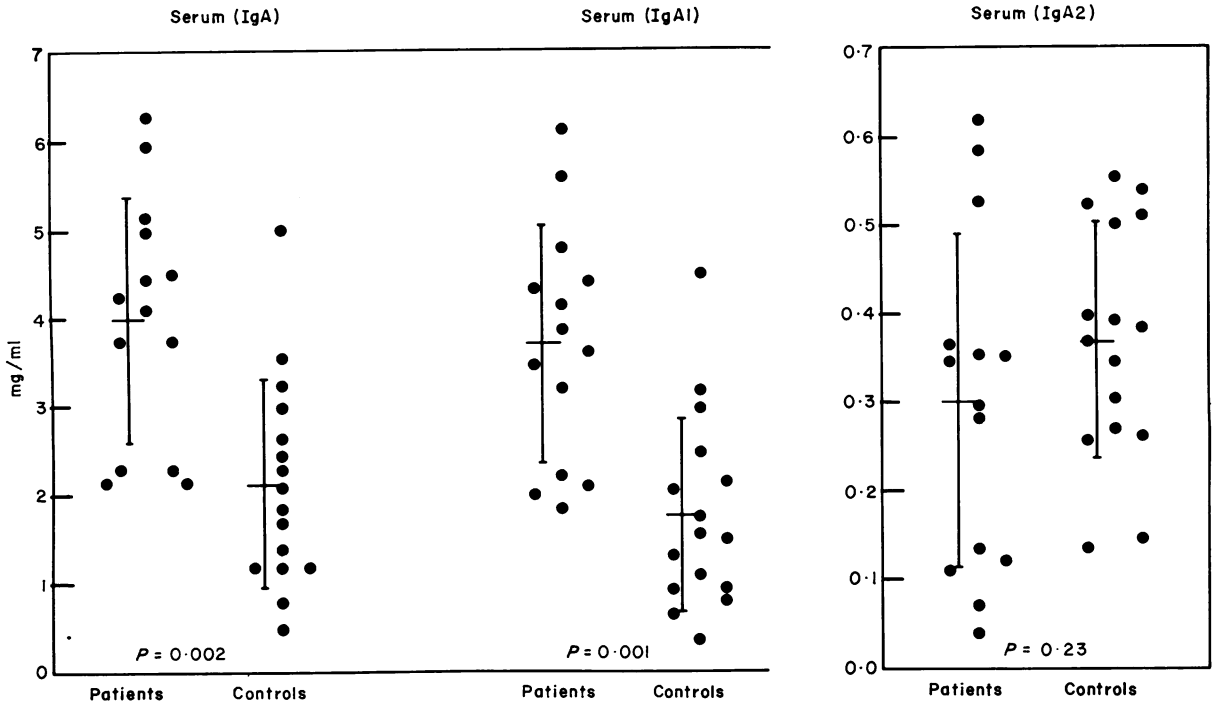

Fig. 1. Serum levels of total IgA, IgA1 and IgA2 in patients and controls (means ± s.d.).

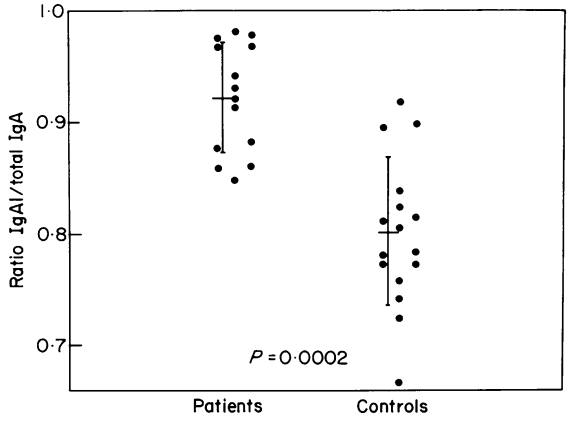

Fig. 2. Ratios of IgA1 to total IgA in sera of patients and controls (means ± s.d.).

controls (92·2 ± 4·9 versus 80·2 ± 6·6%, $P < 0·001$) (Fig. 2). The concentration of serum IgA2 is not different in patients compared to controls (0·30 ± 0·19 versus 0·37 ± 0·13 mg/ml, $P = 0·23$).

Studies of in vitro *immunoglobulin production*

The results for the various isotypes are given in Table 1. Spontaneous production of IgM was significantly higher in patients than in controls ($P < 0·05$). PWM stimulated IgM production was not significantly different from controls ($P = 0·11$). Similarly, no differences were found in IgG or total IgA production, both spontaneous or PWM stimulated.

Whereas IgA1 synthesis was not significantly different from controls, IgA2 synthesis was significantly lower in patients, both in unstimulated cultures ($P < 0·01$), and in PWM stimulated cultures ($P < 0·01$). IgA1 synthesis expressed as a fraction of total IgA synthesis was significantly higher in patients than in controls, again both in unstimulated cultures ($P < 0·05$) and in PWM stimulated cultures ($P < 0·01$) (Fig 3).

Table 1. Production of immunoglobulins by peripheral blood mononuclear cells. (All data in ng/culture ± s.d.)

Isotype	Patients	Controls	*P*
IgM			
Spontaneous	771 ± 548	504 ± 749	0·03
PWM	3973 ± 3534	3203 ± 3684	0·11
IgG			
Spontaneous	222 ± 381	176 ± 91	0·19
PWM	1213 ± 1095	1526 ± 1371	0·65
IgA			
Spontaneous	184 ± 300	134 ± 88	0·27
PWM	604 ± 634	918 ± 845	0·21
IgA1			
Spontaneous	158 ± 278	81 ± 62	0·80
PWM	475 ± 543	523 ± 532	0·74
IgA2			
Spontaneous	26 ± 26	53 ± 32	0·009
PWM	129 ± 182	395 ± 392	0·008
Ratio IgA1/IgA			
Spontaneous	0·75 ± 0·18	0·60 ± 0·17	0·03
PWM	0·75 ± 0·16	0·54 ± 0·19	0·004

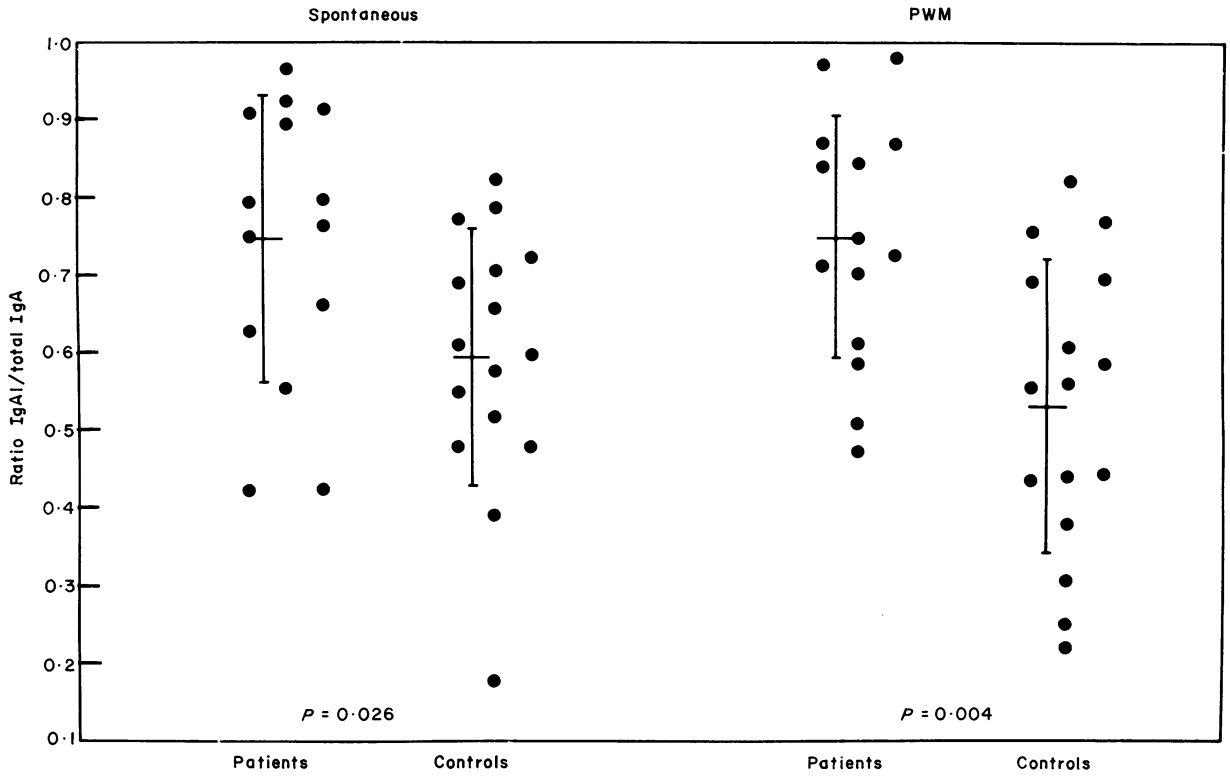

Fig. 3. Ratios of IgA1 to total IgA produced in peripheral blood mononuclear cell cultures, both unstimulated and PWM stimulated (means ± s.d.).

Studies of polymeric IgA produced in vitro

The concentration of SC binding IgA in unfractionated PWM stimulated cultures was not higher in patients (1,083 ± 887 ng/culture) than in controls (1,547 ± 1,488) ($P = 0{\cdot}20$). Likewise, as assessed by HPLC analysis, polymeric IgA as a fraction of total IgA produced was not increased ($P = 0{\cdot}55$) in patients (77·5 ± 20·8%) compared with controls (83·2 ± 9·2%). Similar results were obtained for the fraction of polymeric IgA1 in culture supernatants from patients (74·6 ± 20·4%) and controls (75·9 ± 10·5%) ($P = 0{\cdot}45$).

DISCUSSION

This study demonstrates abnormalities in the serum immunoglobulins and in the production of immunoglobulins by peripheral blood mononuclear cells from patients with IgA nephropathy. The increase in total serum IgA is not a novel finding, and has been previously reported by several investigators (Clarkson *et al.*, 1977; van der Peet *et al.*, 1977; Woodroffe *et al.*, 1980; Mustonen *et al.*, 1981; Lesavre, Digeon & Bach, 1982; Delacroix *et al.*, 1983; D'Amico *et al.*, 1985). We demonstrate that the increase in serum IgA is restricted to the subclass IgA1, and that serum IgA2 concentrations are not different from controls. The ratio of serum IgA1 concentrations to total serum IgA concentrations is increased in patients with IgA nephropathy. Such differences were found earlier in a small group of patients, but were not found to be statistically significant (Delacroix *et al.*, 1983).

Our study of the immunoglobulin production by peripheral blood mononuclear cells yields similar abnormalities since the ratio of IgA1 production to total IgA production is significantly increased in patients with IgA nephropathy. IgA2 production is significantly lower in patients than in controls. We do not confirm the increase in total IgA production by peripheral blood mononuclear cells previously reported, nor do we find an absolute increase in IgA1 production. Normal IgA production has been found by other investigators (Cosio *et al.*, 1982; Rothschild & Chatenoud, 1984; Linné & Wasserman, 1985; Lozano, Garcia-Hoyo & Egido, 1987; Lai *et al.*, 1987). The reason for the significant decrease in the production of IgA2 in patients with IgA nephropathy found in this study is not clear. The mechanisms regulating the synthesis of both IgA subclasses in man are not known, but the available data suggest independent regulation of IgA1 and IgA2 synthesis (reviewed in Kett *et al.*, 1986). A possible explanation for our present findings is that in patients with IgA nephropathy isotype switching occurs preferentially from IgM to IgA1. The impaired synthesis of IgA2 could be the result of this predominant switching to IgA1.

In the interpretation of the apparent discrepancy between the increased serum levels of IgA1, and the normal amounts of IgA1 synthesized by peripheral blood mononuclear cells, it should be remembered that the peripheral blood lymphocytes are not involved in the production of plasma immunoglobulins. Existing data indicate that the majority of circulating B lymphocytes destined to produce IgA will home to the mucosal tissues (Kutteh *et al.*, 1980). The IgA produced in the mucosae is predominantly transported across the epithelial surface and excreted. Only a minority of the circulating B cells will home to the bone marrow. Circulating IgA is predominantly produced by plasma cells in the bone marrow (reviewed in Conley & Delacroix, 1987). It is therefore not surprising that IgA subclasses in serum and in supernatants of peripheral blood

mononuclear cell cultures can be discrepant.

Using two independent methods to determine polymeric IgA, we fail to confirm the previously reported increase in IgA polymer production by peripheral blood lymphocytes *in vitro* (Egido *et al.*, 1982). Even when limited to the relevant subclass IgA1, no difference between patients and controls is found. The high proportion of polymeric IgA produced by PWM stimulated peripheral blood lymphocytes from healthy donors has been previously reported by other investigators (Kutteh *et al.*, 1980; Moldoveanu, Egan & Mestecky, 1984). This observation is consistent with the concept that these cells belong predominantly to the mucosal compartment. In addition to our finding of a normal polymeric IgA production by peripheral blood mononuclear cells there are the previous reports by several groups of normal serum levels of polymeric IgA (Woodroffe *et al.*, 1980; Lesavre *et al.*, 1982; Delacroix *et al.*, 1983). The importance of polymeric IgA in the pathogenesis of the disease remains uncertain in our view. It has been suggested that the discrepant results in the literature are due to the fact that in some studies patients were in an active phase of the disease, whereas in others the patients were examined during an infection-free interval (Feehally *et al.*, 1986). Previously published results concerning IgG and IgM production have been conflicting. In the present study an increased production of IgM was observed.

Our finding of an increased ratio of IgA1 to total IgA in the sera of patients with IgA nephropathy is in agreement with a study we performed recently on the bone marrow of such patients (Van den Wall Bake *et al.*, 1988). In this study a significant increase of IgA1 producing plasma cells was found, with a corresponding increase of the ratio of IgA1 to total IgA produced in culture. Recently a similar increase in IgA1 plasma cells has been noted in the tonsils of patients with IgA nephropathy (Nagy & Brandtzaeg, 1988). Combining the results of these studies we conclude that patients with IgA nephropathy develop an antibody response, both at mucosal sites and systemically, characterized by a shift in the subclass distribution toward IgA1. Ongoing or repeated stimulation at mucosal surfaces could result in activation and proliferation of B lymphocytes destined to differentiate into IgA1 producing plasma cells. These B lymphocytes enter the circulation and either return to the lamina propria of mucosal sites or localize in the bone marrow. The existence of such a mucosal-bone marrow axis has recently been demonstrated in mice (Alley, Kiyono & McGhee, 1986). These bone marrow plasma cells are responsible for long term overproduction of IgA1, presumably specific antibodies, leading to increased serum concentrations. It is very likely that part of these IgA1 antibodies will ultimately localize in the mesangium of glomeruli by an unknown mechanism.

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