Effect of treatment with 17 α -alkylated androgens on C4 conversion products in hereditary angioedema studied by crossed immunoelectrophoresis

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SUMMARY During agarose electrophoresis C4 in normal human serum is converted into cleavage products of $\beta 1$ and $\beta 2$ mobility. By contrast in the serum of untreated patients with hereditary angioedema C4 gives only one $\beta 2$ peak on crossed immunoelectrophoresis. The normal C4 electrophoretic pattern is restored in serum of patients treated with stanazolol but not with danazol despite the same C1-esterase inhibitor (C1 INH) activities and C4 serum concentrations. We suggest that stanazolol besides having specific effect on C1 INH activity can interfere with other protease inhibitors affecting C1 activation.

Hereditary angioedema (HAE) is an inherited disorder characterised by a decreased functional activity and/or antigenic concentration of C1-esterase inhibitor (C1 INH) which induces a constant activation of C1 leading to the cleavage of its natural substrates C4 and C2 which are found at low concentration in the serum of HAE patients.¹

Treatment with 17 α -alkylated androgens (danazol and stanazolol) induces clinical remission and corrects the biochemical defect. During androgenic treatment C1 INH functional activity increases up to 50% of normal value and C4 serum concentration is normalised.² After a course of stanazolol C4 antigenic concentration is usually higher than after danazol treatment.³ The visualisation of C4 conversion products is useful in the study of in vivo complement activation.^{4,5} The crossed immunoelectrophoretic (CIE) analysis of C4 both with EDTA-plasma or purified protein shows a single peak in $\beta 2$ region; when normal human serum (NHS) is used, an additional peak in $\beta 1$ region is produced representing the C4 cleavage product (C4b complexed to C4 binding protein) resulting from C1 activation probably during electrophoresis.⁶ In presence of C4 binding protein (C4bp) and C3b inactivator (C3bi-NA), C4b in fluid phase is dissociated to form two distinct fragments C4c (α mobility) and C4d (β mobility).^{7,8} Different behaviour of C4 on CIE has been observed in the serum of the patients with HAE on

and off danazol and stanazolol treatment: these observations are reported here.

Materials and methods

NORMAL AND PATHOLOGICAL SPECIMENS

Blood was drawn into plastic tubes without anti-coagulant or containing 0.01 M sodium-EDTA (EDTA). Centrifugation was carried out within two hours at +4°C and samples were stored in small aliquots at -30°C. Serum and EDTA-plasma samples were taken from 10 healthy adult volunteers and from 10 HAE patients. Samples from 10 HAE patients were obtained on and off treatment with danazol (400-600 mg/day for one month) and in two of these patients samples were obtained on and off treatment with stanazolol (4-6 mg/day for one month). At the sampling time all the patients were asymptomatic. All the samples were analysed by CIE within three hours and after one month at -30°C.

CROSSED IMMUNOELECTROPHORESIS

Crossed immunoelectrophoresis (CIE) was performed according to the method of Laurell on Bar-bital buffer pH 8.6, ionic strength 0.02. The electrophoresis was carried out at 10 V/cm for 120 min in 0.9% agarose (Behringwerke AG, Marburg/Lahn, W Germany) in electrophoretic buffer cooled at 10°C. Under these conditions albumin, marked with bromophenol blue, moved about 9 cm from the origin. Second dimension runs were performed

using potentials of 1–2 V/cm overnight. The gel plates were washed and stained with Coomassie Brilliant Blue.

Radial immunodiffusion (RID)

C1-esterase inhibitor and C4 serum concentrations were determined by RID on cellulose acetate strips.⁹

C1 INH functional activity

C1-esterase inhibitor activity was performed according to the method of Lachmann.¹⁰

Antisera

Monospecific antisera to C1 INH (Behringwerke AG, Marburg/Lahn, W. Germany) and to C4 (DAKO-immunoglobulins, Copenhagen, Denmark) were used.

C1 INH and C4 levels in normal subjects

C1-esterase inhibitor and C4 concentration were determined in 39 healthy adult volunteers (20 males and 19 females);

C1 INH (mean $\pm$ SE) serum concentration:

99.46 $\pm$ 2.71% of normal standard pool

C1 INH (mean $\pm$ SE) activity:

99.25 $\pm$ 1.38% of normal standard pool

C4 (mean $\pm$ SE) serum concentration:

27.52 $\pm$ 0.69 mg/100 ml.

Results

Serum and EDTA-plasma analysed by CIE in normal subjects always gave the classically described patterns (Fig. 1). If EDTA was added to NHS

immediately before electrophoresis, C4 gave only one peak in β 2 region and in some agarose plates a minor peak in α region was observed. EDTA-plasma or serum from 10 HAE untreated patients (C4 serum concentration less than 8 mg/100 ml and C1 INH functional activity and immunological concentrations less than 15% of normal standard pool) gave only one β 2 peak, as observed in normal EDTA-plasma, and a less reproducible minor peak in α region. We never observed a peak in β 1 region as described for NHS (Fig. 2).

The same patients were treated with danazol for one month: C1 INH immunological concentrations and functional activity increased to 30–70% and to 35–50% of normal standard pool, respectively, C4 serum concentration also increased to 25–30 mg/100 ml. In these patients EDTA-plasma and serum analysed by CIE gave one peak in β 2 region and one minor peak in α but nothing in β 1 region. Danazol treatment was then withdrawn and C1 INH and C4 concentrations returned in six days to pretreatment values.

Two patients (FC, PG) one month after withdrawal of danazol, were then treated with stanazolol. In patient FC after three weeks of stanazolol C4 concentration increased again to 37 mg/100 ml and C1 INH immunological concentration and activity to 40% and 50% of normal standard pool, respectively. After four weeks the values were 43 mg/100 ml, 50% and 65%, respectively. Similarly, in patient PG after three and four weeks of stanazolol treatment C4 concentrations were 29–30 mg/100 ml, C1 INH immunological concentrations and activity were 40–45% and 40–50% of

Fig. 1 Crossed immunoelectrophoretic pattern of C4 in normal human EDTA-plasma (a, b) and in corresponding serum (c, d). Serum C4 is cleaved in two fractions. Vertical lines indicate the position of β 1 and β 2 bands of the corresponding agarose electrophoresis.

normal standard pool, respectively. The CIE analysis of these EDTA-plasma and serum samples reproduced the normal pattern giving the single β_2 peak with EDTA-plasma and the bimodal distribution with serum; an α minor peak was present as usual (Fig. 3). No differences in electrophoretic pattern were obtained with fresh or frozen samples of normal and pathological subjects.

Discussion

Danazol and stanazolol can be used in HAE without significant differences: in fact both drugs induce clinical remission, normalise serum C4 concentration and increase functional and immunological C1 INH to 50–65% of the normal value. However, we found

that during stanazolol treatment C4 concentrations were in some patients significantly higher than with danazol.³ The hypothesis that this effect could be a result of a reduced activation of C1 by a stronger action on C1 INH seems unacceptable. In fact C1 INH was found at the same level with both drugs and furthermore in untreated HAE patients C1 functional activity is often normal.^{11–13} We therefore investigated other possibilities by considering the CIE patterns of C4.

We found that the different behaviour of C4 in normal sera (bimodal distribution) versus EDTA-plasma (one major β_2 peak) was absent in HAE patients (only one major β_2 peak). However, the bimodal distribution can be restored during stanazolol but not danazol treatment. This discre-

Fig. 2 Crossed immunoelectrophoretic pattern of C4 in serum (a, b) and in corresponding EDTA-plasma (c, d) of HAE patients: in serum β_1 fraction is absent.

C4 antigen level = 4 mg/100 ml (a); 6 mg/100 ml (b). C1 INH activity (% of normal standard pool) = 6% (a); 12% (b). C1 INH antigen level (% of normal standard pool) = 7% (a); 10% (b).

Fig. 3 Effect of danazol and stanazolol on C4 in serum of two patients analysed by CIE: pretreatment condition (a, b); one month danazol treatment (c, d); one month stanazolol treatment (e, f). Serum β_1 fraction appears only after a course of stanazolol. C4 antigen levels (mg/100 ml): (a) 6; (b) 4; (c) 30; (d) 25; (e) 30; (f) 37. C1 INH activity (% of normal standard pool): (a) 10; (b) 8; (c) 50; (d) 45; (e) 40; (f) 45.

C1 INH antigen level (% of normal standard pool): (a) 12; (b) 10; (c) 48; (d) 40; (e) 45; (f) 50.

pancy could be seen at the same C4 and C1 INH serum levels: more specifically C4 never split in spite of reaching normal levels after danazol treatment.

Previous work⁶ showed that the different C4 electrophoretic patterns in serum and EDTA-plasma reflects the C1 activation during agarose electrophoresis and the binding of C4 activated (C4b) to C4 binding protein (C4bp).¹⁴ In HAE serum with low concentrations of C4 the bimodal distribution does not occur probably because C4bp is not available to complex with C4b during C1 activation by agarose. The failure of danazol to restore the normal electrophoretic pattern suggests that the uncontrolled complement activation in HAE is probably not only related to C1 INH deficiency. In fact the higher C4 concentrations reached with stanazolol and its capacity to restore physiological C4 activation, seen by CIE, lead us to suggest an additional action of stanazolol other than on C1 INH. This may be on other protease inhibitors interfering with complement activation and in particular restoring the optimal ratios of C4b, C4bp and C3bINA, the factors involved in fluid phase cleavage of C4b.

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