Relationship between angiotensinogen, α_1-protease inhibitor elastase complex, antithrombin III and C-reactive protein in septic ARDS

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Summary. The time-course of plasma angiotensinogen (Ao), elastase-α_1-protease inhibitor complex (ELα_1PI), antithrombin III (AT III) and C-reactive protein (CRP) have been investigated of six patients suffering from adult respiratory distress syndrome (ARDS).

The total plasma Ao level (active and inactive Ao) varied in individuals but was increased up to five-fold. An increasing amount of inactive Ao is found. From the beginning of their stay in the intensive care unit up to five days half of the patients displayed a positive correlation between the plasma CRP and Ao level. The CRP and Ao values were either not or were negatively correlated with the AT III values. In contrast plasma Ao and AT III levels in all patients were positively correlated during a particular period in the subsequent phase of the disease, where there was no or a negative correlation with CRP. The two acute phase reactants CRP and ELα_1PI were only correlated in two patients at the beginning of the disease.

The markedly increased plasma level at the beginning of the inflammatory disease indicates that Ao is an acute phase reactant, and this is supported by the parallel changes in plasma CRP and Ao levels during the early days of ARDS. The relationship between the plasma levels of Ao and AT III for more than fourteen days suggests similar regulation of these members of the serpin family after termination of the acute-phase.

Key words: angiotensinogen, antithrombin, acute-phase proteins, septic ARDS; elastase-α_1-protease inhibitor complex, C-reactive-protein, renin-angiotensin system, adult respiratory distress syndrome, sepsis

Angiotensinogen (Ao) is the only known precursor of the vasoactive peptide hormone angiotensin. This plasma glycoprotein is synthesized and secreted by the liver. Various hormones are known to be involved in its synthesis and secretion, including angiotensin II (Khayyal et al., 1973), oestrogens and glucocorticoids (Crane et al., 1971), and thyroid hormones (Dzau and Herrmann, 1982). Subsequent to publication of the cDNA sequence of Ao by Ohkubo and coworkers (1983), a sequence relationship to the serpin (serine protease inhibitors) family, antithrombin III (AT III), ovalbumin and expecially to α_1-protease inhibitor (α_1PI) was detected by Doolittle (1983). This was supported by the finding of Tanaka et al. (1984), showing a common structural organization of the Ao and α_1PI genes. α_1PI interacts with lysosomal enzymes, including kallikrein, various cathepsins and especially elastase to form the elastase-α_1-protease inhibitor complex (ELα_1PI). A III is the most important inhibitor of the clotting system (Travis and Salvesen, 1983). AT III and α_1PI are acute-phase reactants in various animals (Koj and Regoeczi, 1978; Kushner, 1982), whereas in humans A III is not (Martínez-Brotóns et al., 1987). C-reactive protein (CRP) is a further acute-phase reactant, which does not belong to the serpin family. The physiological role of CRP is not yet comletely understood (McCarty, 1982). Recent data have shown that CRP-peptides are potent immunomodulators (Robey et al., 1987). Within hours following acute inflammation, the synthesis of these proteins is increased in the liver and their plasma concentration is elevated by as much as 2000-fold (Koj and Gordon, 1983; Morley and Kushner, 1982). It has recently been shown that administration of lipopolysaccharides (LPS) to rats results in a marked increase in α_1PI and Ao mRNA in the liver (Kageyama et al., 1983) and an increase in plasma Ao levels (Hilgenfeldt, 1988). These findings indicate that Ao is a member of the acute-phase reactant class in rats. To verify its role in inflammatory diseases in men, the plasma levels of Ao have been compared over a 4 week period with those of ELα_1PI, AT III and CRP in patients suffering from septic ARDS (Modig, 1986).

Materials and methods

Patients

Plasma samples from six patients (3 male, 3 female) with a mean age of 33 (18.5, SD) y and a mean weight of 67.7 kg, admitted to the Intensive Care Unit, Klinikum Großhadern, University of Munich

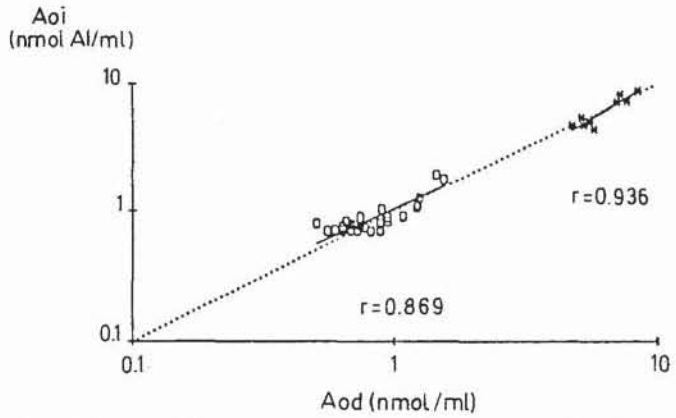

Fig. 1. Plasma angiotensinogen in control group obtained by direct (Aod) and indirect (Aoi) radioimmunoassay (centre group), $n = 24$. Mean (SD) 0.88 (0.24) nmol Ao/ml. Plasma angiotensinogen in pregnant women (right group), $n = 12$, 6.2 (1.3) nmol Ao/ml. The solid line is the calculated correlation between the assays and the dotted line shows the theoretical 1:1 correlation

were investigated. Clinical data are given in Table 1. Only patients kept in the Intensive Care Unit for more than 10 days were eligible for the study. These critically ill patients all suffered from bacterial sepsis and the adult respiratory distress syndrome (ARDS) (Modig, 1986).

All patients received antibiotics, low dose heparin, diazepam, morphine and pancuronium bromide. In addition, furosemide was administered if urine flow was reduced. Epinephrine and dopamine were administered to five of the six patients (except P 10) to maintain blood pressure and kidney function.

Criteria for exclusion. Patients were excluded in cases of negative blood culture or no focus of severe infection. Patients with liver failure evidenced by history and laboratory tests, with neoplastic disease, with any chronic illness, or who received glucocorticoids or oral contraceptives were also excluded.

Preparation of plasma. Up to four blood samples per day were obtained from indwelling venous catheters. Blood was collected in 5 ml plastic tubes containing 0.5 ml trisodium citrate 0.13 M. Plasma was prepared by centrifugation of citrated blood at 1000 g for 10 min at room temperature and frozen at −70°C in plastic tubes.

Assay of angiotensinogen. Ao was completely purified from plasma obtained from the Department of Gynaecology, University of Heidelberg by a five step procedure. Purity was verified by amino acid analysis, end group sequence analysis and complete liberation of N-terminal angiotensin I by renin. The protein had a specific activity of 21.1 µg ANG I · mg^{-1} protein. Asuming a molecular weight of 60,000 Da for human angiotensinogen, the preparation has a purity of 98% (Kienapfel, 1987). Antibodies against human Ao were raised in rabbits by the method of Vaitukaitis et al. (1971). Plasma Ao concentrations were determined by a direct radioimmunoassay (RIA) performed according to the method described for rat Ao (Hilgenfeldt and Schott, 1987). Antiserum was used at 1:25 000. The sensitivity of the assay was 1 fmol Ao. In addition, an indirect assay following enzymatic cleavage of Ang I with an excess of renin (Hilgenfeldt and Hackenthal, 1979), was also used. Ang I was measured by RIA (Menard and Catt, 1972). Ang I antiserum was raised in rabbits using glutaraldehyde-linked angiotensin I. The serum was analyzed for cross-reactivity with Ang II. The antibody titre for Ang I and Ang II differed by 5.10^4.

Assay of elastase-α_1-proteinase inhibitor complex (Elα_1PI). ELα_1PI complex was measured using a commercial, solid phase, enzyme-linked, sandwich immunoassay (E. Merck AG, Darmstadt, FRG), as described by Jochum et al. (1983). The range was from 20 to 150 µg α_1PI-complex per litre.

Assay of plasma antithrombin (AT III). Plasma AT III concentration was determined with a commercial thrombin inhibition assay (S-2238, Deutsche Kabi-Vitrum, Munich) using p-nitroaniline as the cromogenic product.

Assay of C-reactive protein (CRP). The concentration of C-reactive protein was measured by radial immunodiffusion using a commercial test system (Behring Werke AG, Marburg, FRG). The coefficient of correlation was calculated by comparing different parameters of every plasma sample of each patient using the computer program Microsoft Chart.

Results

Control plasma Ao levels

The values obtained by the direct and indirect assays for Ao were compared by measuring the angiotensinogen content in plasma from 25 healthy volunteers. The plasma Ao levels were 0.876 (0.278) nmol Ao · ml^{-1} and 0.874 (0.237) nmol Ang I · ml^{-1} (mean (SD). This indicates that in healthy man the plasma Ao fluctuates within a narrow range. In addition plasma from 10 healthy pregnant women, with the pregnancy dependent elevation of Ao was also tested. The mean plasma Ao was 6.19 (1.25) nmol Ao · ml^{-1} and 6.07 (1.6) nmol Ang I · ml^{-1}. As shown in Fig. 1, the correlation coefficient between the direct and indirect assays for Ao in both groups was close to 1.

Patients

Ao, ELα_1PI, AT III and CRP were analyzed in 209 different plasma samples from 6 patients. As shown in Fig. 2, plasma Ao followed an unique time course in each pa-

Table 1. a) Traumatic event (days prior admission; Day 0 = beginning of the study, Day of admission to the intensive care unit; m = male, f = female) b) Clinical diagnosis ($n = 6$)

P 9	a) 10 (f), near drowning b) Severe ARDS two days prior admission, *Legionella pneumophilia*, sepsis syndrome on Days 8–10 of the study with acute renal insufficiency, rhabdomyolysis and death on Day 27.
P 10	a) 5 (m) polytrauma. b) Pneumonia, sepsis *(Staph. aureus, Ps. aeruginosa)*, ARDS. On Day 6 acute renal insufficiency. Second infection on Day 16.
P 17	a) 8 h (m) Polytrauma and severe posttraumatic ARDS. b) At Day 10 pneumonia, at Day 18–20 sepsis and acute renal insufficiency.
P 45	a) 3 (f), sepsis syndrome 6 h prior admission. b) Shock followed by severe ARDS, recurrent infections (pneumonia, urinary tract infections). On Day 3 acute renal insufficiency.
P 48	a) 0 (f), hysterectomy. b) postoperative sepsis *(Cl. perfringens)*, severe consumptive coagulopathy. Day 3 re-laparotomy
P 53	a) 7 (m), polytrauma. b) Sepsis, severe ARDS required artificial respiration for 24 days. Day 2 acute renal insufficiency.

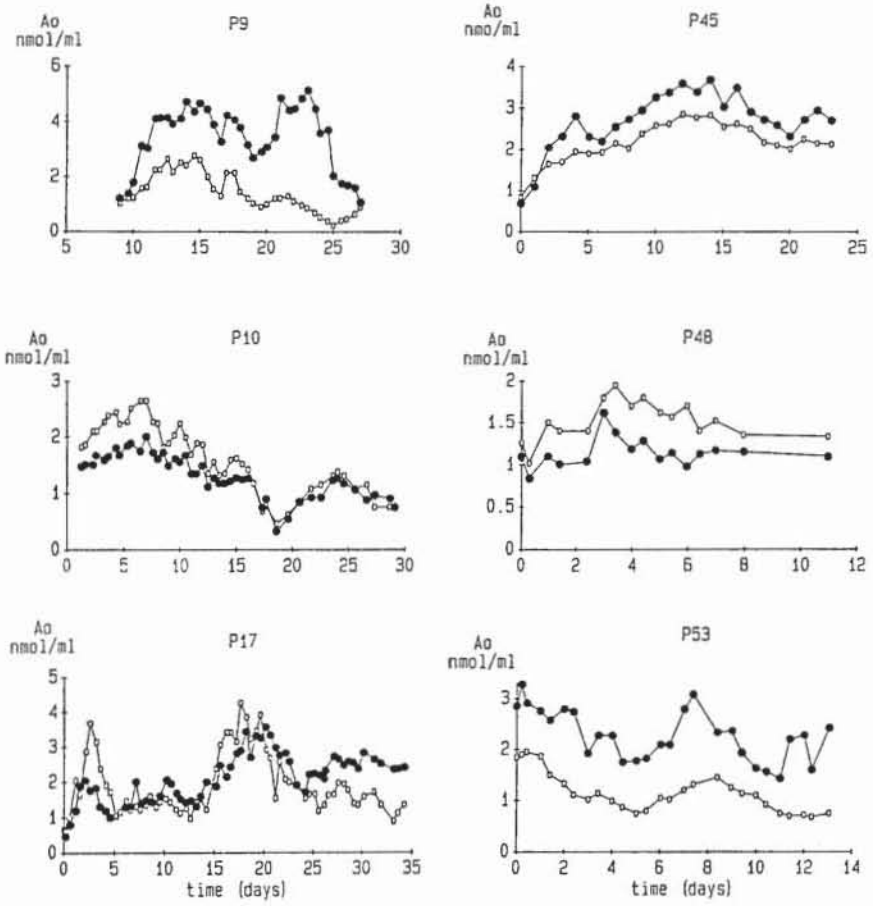

Fig. 2. Plasma Ao levels (nmol/ml) obtained by the direct (solid symbols) and indirect (open symbols) radioimmunoassay (nmol A I/ml) in six patients with septic ARDS

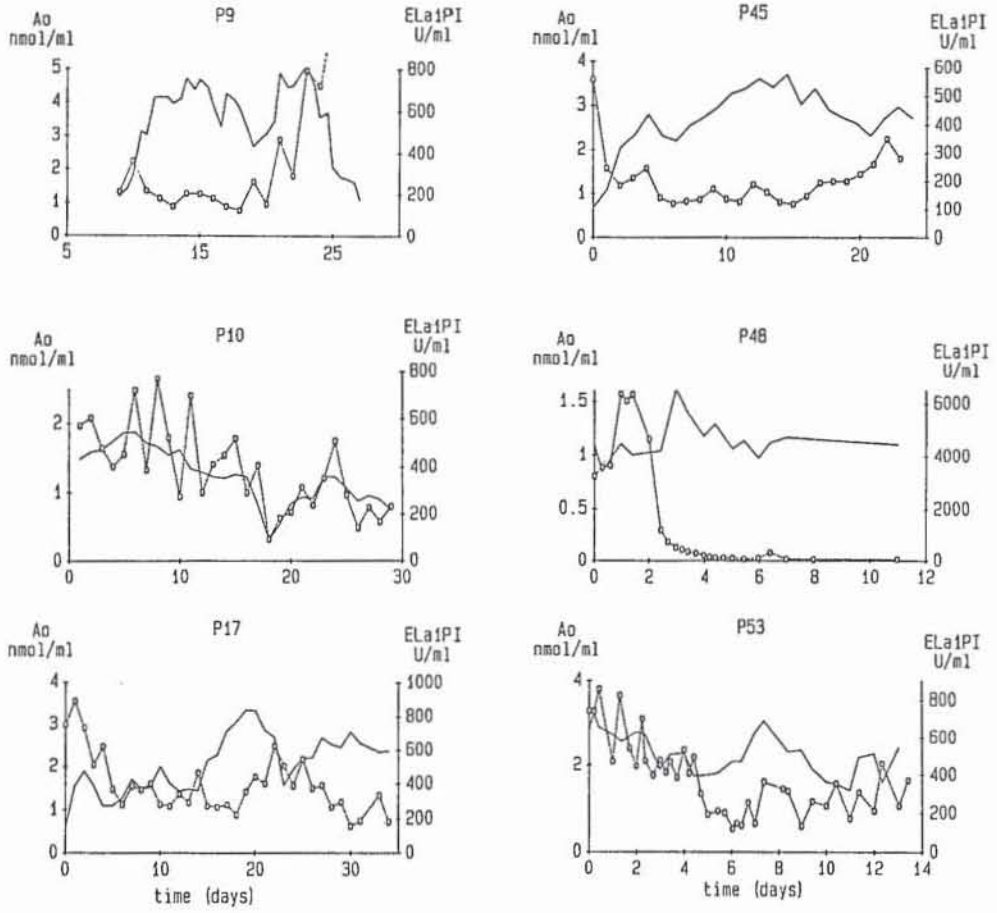

Fig. 3. Plasma Ao and ELα_1PI levels in six patients suffering from sepsis. The Ao values in the direct radioimmunoassay are consistent with those of Fig. 2. (Ao solid line; ELα_1PI open symbols)

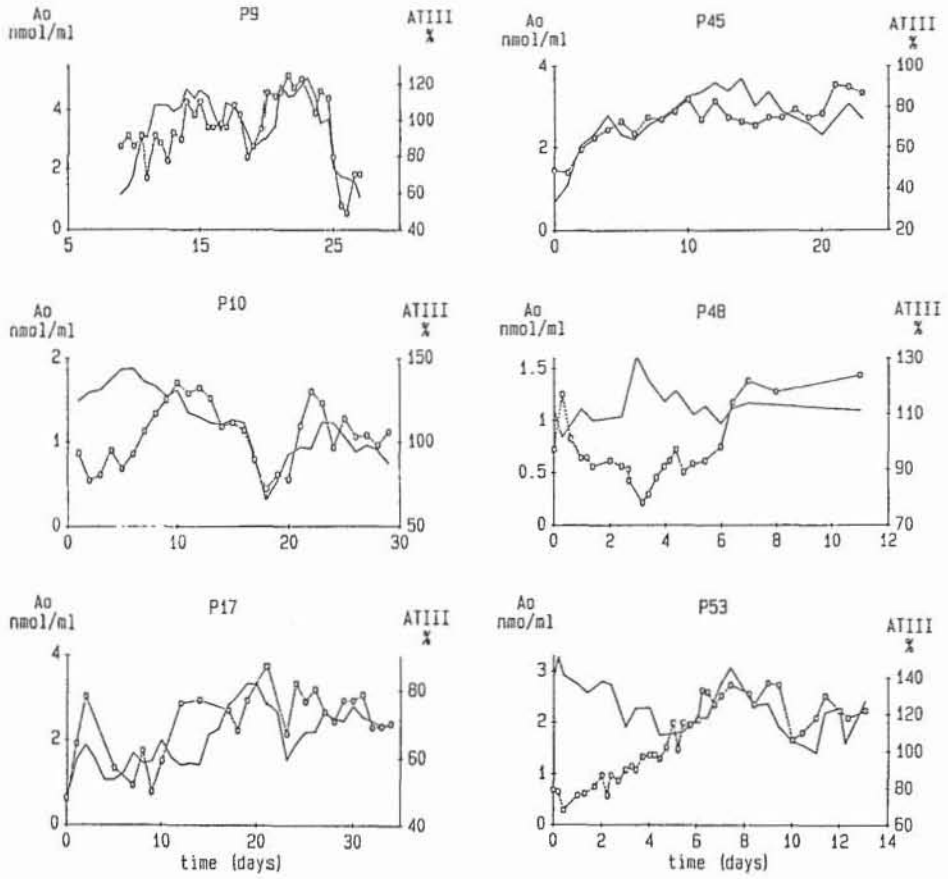

Fig. 4. Plasma Ao and AT III levels in six patients suffering from sepsis. The Ao values in the direct radio-immunoassay are consistent with those of Fig. 2 (Ao solid line; AT III open symbols)

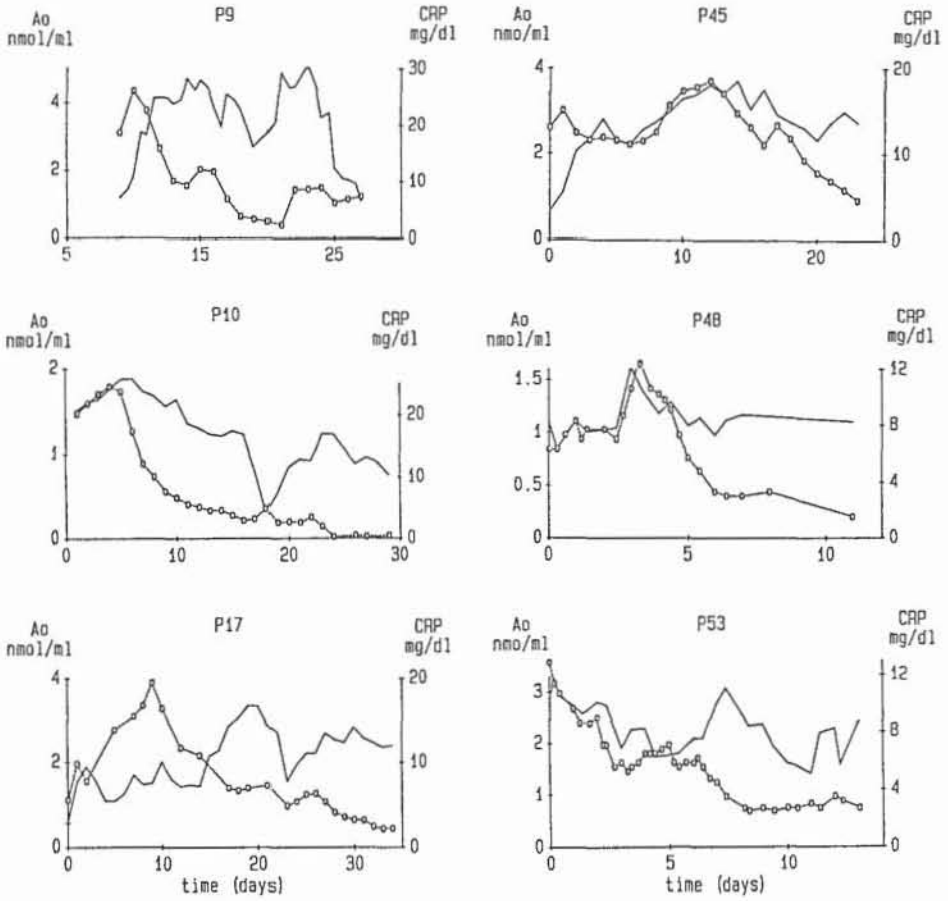

Fig. 5. Plasma Ao and CRP levels in six patients suffering from septic ARDS. The Ao values in the direct radio-immunoassay are consistent with those of Fig. 2 (Ao solid line; CRP open symbols)

Table 2. Correlation coefficient (r) of plasma Ao levels obtained by direct (d) and indirect (i) RIA and corresponding plasma ELα_1PI, AT III and CRP in six patients suffering from septic ARDS. The time-period after admission to the hospital (days) and as the isolated values (n) during this period are given. Values in parentheses are the correlation coefficients for limited time periods

Patient No	9	10	17	45	48	53
Aod-Aoi	0.51 (0.814) {0.489}	0.96 (0.758) {0.955}	0.649	0.932 (0.967) {0.578}	0.709 (0.827) {−0.583}	0.74 0.876) {0.474}
Aod-ELα_1PI	0.500 (−0.479) {−0.648}	0.478 (−0.737) {0.438}	−0.580	−0.587 (−0.699) {−0.135}	−0.357 (−0.509) {−0.791}	0.489 (0.734) {−0.123}
Aod-AT III	0.69 (0.468) {0.884}	0.038 (−0.476) {0.728}	0.709	0.711 (0.851) {−0.096}	−0.39 (−0.679) {0.869}	−0.351 (−0.636) {0.715}
Aod-CRP	−0.202 (−0.461) {−0.246}	0.494 (0.778) {0.045}	−0.294	0.213 (0.567) {0.237}	0.531 (0.789) {0.107}	0.579 (0.784) {0.144}
AT III-CRP	−0.303 (−0.483) {−0.434}	0.488 (−0.402) {0.323}	−0.417	−0.190 (0.354) {−0.711}	−0.793 (−0.617) {−0.794}	−0.825 (−0.706) {0.070}
ELα_1PI-CRP	−0.171 (0.685) {0.235}	0.351 (−0.531) {0.405}	0.083	−0.300 (−0.233) {−0.667}	0.233 (−0.350) {0.307}	0.736 (0.634) {−0.499}
time after admission (days)	9–27 (9–18.6) {19–27}	1.3–29 (1.3– 5.3) {9 –29}	0–34.2	0–23.7 (0–14) {14–24}	0–11 (0– 5) {5–11}	0–13 (0– 5) {5–13}
n	37 (20) {17}	46 (9) {31}	35	49 (30) {19}	16 (10) {6}	26 (12) {14}

tient. The amount of Ao in all patients was increased at some time during the period of observation. In addition, the values obtained by the direct and indirect measurement show marked differences. In contrast to the control data given in Fig. 1, the two assay systems did not show a 1:1 ratio in plasma from these patients, although the values were still correlated (Table 2).

Within a certain period, the values obtained with the direct assay were significantly higher in P9, P17, P45 and P53. This indicates increased formation of Ang I, leading to an increased plasma concentration of des-Ang I-Ao. Stimulation of the components of the renin-angiotensin system, e.g. Ao, renin, Ang I and Ang II, in some of these patients has recently been reported (Hilgenfeldt et al., 1987). The Ao values obtained by the direct assay were compared with the levels of ELα_1PI, AT III and CRP. Plasma Ao and ELα_1PI in all six patients are shown in Fig. 3. A positive correlation of Ao and ELα_1PI was not observed in most patients. Only in P10 was there fluctuation of the ELα_1PI values around the Ao values, although they were not positively correlated; in P53 $r = 0.734$ was found within five days after admission.

The plasma levels of Ao and AT III are shown in Fig. 4. A positive correlation of Ao and AT III was evident in plasma from three patients during the entire observation period, with r between 0.69 and 0.71. The other three patients (P10, P48 and P53) show a good correlation within a limited period (Table 2, data in parentheses). α_1PI and AT III are members of the same gene family and so are related to angiotensinogen. However, the acute phase protein CRP does not belong to this group. The plasma levels of Ao and CRP are shown in Fig. 5. It is interesting to note that in P10, P48 and P53 the Ao and CRP values were positively correlated at the beginning of the illness. In the time interval in which plasma Ao and CRP were positively correlated, however, Ao and AT III showed a negative or no correlation (see Table 1). A correlation between CRP and ELα_1PI was less common. In addition, there was no or almost a negative correlation between CRP and AT III in plasma from all the patients.

Discussion

The mean plasma Ao level in healthy volunteers was 0.9 (0.3) μM. An increase in plasma Ao is observed in pregnancy, in women taking oral contraceptives, and in hypertension (Gordon, 1983). The possibility that Ao may behave as an acute-phase protein was first raised by Bing (1972), although he did not find an increase in plasma Ao levels following turpentine injection in the rat. The idea that Ao may be an acute-phase reactant was revived after the finding that Ao was genetically closely related to α_1PI (Doolittle, 1983 and Tanaka et al., 1984). The idea was supported by the finding of as much as a five-fold increase in Ao mRNA in liver (Kageyama et al. 1983) and a three-fold increase in plasma Ao levels (Hilgenfeldt, 1988) following lipopolysaccharide administration to rats. Although these data are consistent with the hypothesis that Ao is an acute-phase reactant in humans, caution is needed in inferring that data from one species can be applied to another; for example, α_1PI is not an acute-phase

reactant in mice (Baumann et al., 1986), and CRP demonstrates striking species variations in response to tissue injury (Pepys et al., 1979).

Nielsen and Knudsen (1987) recently reported a 70% increase in plasma Ao levels in patients with acute inflammatory disease, indicating that Ao may be an acute-phase reactant in man. Since the regulation of plasma Ao levels is complex, an increased level on its own cannot be accepted as proof of this hypothesis. The acute-phase response occurs in the period immediately following injury and infection. Therefore, the present study was designed to follow the time course of change in Ao levels in patients with bacterial sepsis. The changes were compared with those in known acute-phase reactants.

Increased *plasma levels* of Ao were found during the first 3 to 5 days of observation in five of the six patients. The correlation between Ao and the other acute-phase proteins in our study was based on the direct RIA for Ao. This assay recognizes the total Ao concentration and ignores Ao catabolism by plasma renin. In addition, both proteins, intact Ao and des-AngI-Ao, have an almost identical plasma half life in vivo (Hilgenfeldt, 1988) and should be eliminated to a similar extent.

The individual course of the disease in each patient makes it difficult to find a common principle. It is impossible at present to model such complex curves of different plasma parameters, so it is only possible to compare different parameters from each plasma sample and to fit them in a linear correlation. A relationship between *Ao and* α_1*PI* was seen in only one patient during five days after admission (P53; $r = 0.73$). However, the assay for α_1PI only measured the ELα_1PI-complex and not the total amount of α_1PI. Therefore, a correlation between Ao and α_1PI would only have been detected if the amount of the ELα_1PI-complex and of free α_1PI were linked. A far stronger relationship was found between *Ao and AT III*, with a significant correlation during the entire observation period in three patients (P9, P17, P45). In those patients no correlation of Ao and CRP was found. The other three patients, P10, P48, P53, displayed a good correlation between Ao and CRP during the initial part of the study ($r = 0.78$, 0.79, 0.78). During this period both parameters were negatively correlated with AT III (Ao-AT III: $r = -0.48$, -0.68, and -0.64; CRP-AT III: $r = -0.40$, -0.62, -0.71). In the subsequent period Ao and AT III displayed a good correlation ($r = 0.73$, 0.87, 0.72) without being related to CRP.

The good correlation of the plasma levels of Ao and CRP during the first several days of hospitalization suggests that Ao is an acute-phase reactant in man. That this correlation was not found in all six patients was mainly due to the fact that data from the initial acute-phase could only be obtained from one patient (P48). On the other hand, plasma Ao and AT III levels showed a far closer relationship, although AT III is not an acute-phase protein in man (Martínez-Brotóns et al., 1987). However this correlation is observed during a time period when Ao and CRP are not correlated. This indicates that in the latter period the acute-phase has already been terminated. A correlation in the plasma levels of genetically related proteins like Ao, α_1PI and AT III, indicates not only a similar synthetic response to stimuli, but in addition it points to similar rates of *catabolism and elimination*, despite their molecular and functional differences. There is evidence of increased Ao catabolism in acute inflammation, since there is a decrease in the in vivo half-life of one form of Ao in rats following LPS stimulation. Decreased renal elimination, which might be caused by decreased renal blood flow, has also been discussed (Hilgenfeldt, 1988).

At present a common stimulus for acute-phase proteins cannot be defined. Very recently it has been shown that the *synthesis* of various acute-phase proteins is mediated by cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), by a direct action on hepatocytes (Ramadori et al., 1988). However while the secretion of α_2-macroglobulin is markedly accelerated by IL-6, the secretion of cysteine proteinase inhibitor and α_1PI is unaffected (Andus et al., 1988). On the other hand, a macrophage derived factor is able to stimulate the synthesis and secretion both of AT III and α_1PI (Hoffmann et al., 1986). The biosynthesis of the most prominent acute-phase protein CRP by the liver is presumably mediated by interleukin-1 (Kaplan, 1982; Syin et al., 1986 and Goldberger et al., 1987). Very recently it has been shown that the acute-phase response of Ao is mediated by interleukin-6 in rat hepatoma cells (Itoh et al., 1989), but this requires the presence of glucocorticoids, suggesting that the latter are essential for the stimulatory effect.

The role of angiotensinogen in inflammation cannot yet be defined. It appeared from a previous study (Hilgenfeldt et al., 1987) that the renin-angiotensin system did not play a role in the maintenance of blood pressure in such patients. However, angiotensin may be important in the immune response during inflammation (Fernandez-Castello et al., 1987).

In conclusion, up to a five-fold increase in plasma Ao levels has been found in patients suffering from septic ARDS. The positive correlation between Ao and CRP during the first days after admittance to the intensive care unit indicates that Ao is an acute-phase reactant in man. There was also a good correlation between plasma Ao and AT III levels during the later stage of disease, suggesting similarities in the synthesis and elimination kinetics of these proteins. Further studies are required to define the functional role of Ao in inflammatory diseases.

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