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CLINICAL STUDY

Mechanism of inhibition of cytochrome P450 C21 enzyme activity by autoantibodies from patients with Addison's disease

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Abstract

Objective: To study possible mechanisms for the inhibition of cytochrome P450 C21 (steroid 21-hydroxylase) enzyme activity by P450 C21 autoantibodies (Abs) *in vitro.*

Design: Two possible mechanisms for the inhibition of P450 C21 enzyme activity by P450 C21 Abs were studied: (a) conformational changes in the P450 C21 molecule induced by Ab binding and (b) the effects of Ab binding to P450 C21 on the electron transfer from the nicotinamide adenine dinucleotide phosphate reduced (NADPH) cytochrome P450 reductase (CPR) to P450 C21.

Methods: The effect of P450 C21 Ab binding on the conformation of recombinant P450 C21 in yeast microsomes was studied using an analysis of the dithionite-reduced CO difference spectra. The effect of P450 C21 Abs on electron transfer was assessed by analysis of reduction of P450 C21 in the microsomes in the presence of CO after addition of NADPH.

Results: Our studies confirmed the inhibiting effect of P450 C21 Abs on P450 C21 enzyme activity. Binding of the Abs did not induce significant change in the P450 C21 peak at 450 nm (native form) and did not produce a detectable peak at 420 nm (denatured form) in the dithionite-reduced CO difference spectra. This indicated that conformation of P450 C21 around the heme was not altered compared with the native structure. However, incubation of the P450 C21 in yeast microsomes with P450 C21 Ab inhibited the fast phase electron transfer from the CPR to P450 C21.

Conclusions: Our observations suggested that the mechanism by which P450 C21 Abs inhibit P450 C21 enzyme activity most likely involves inhibition of the interaction between the CPR and P450 C21.

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Introduction

Autoimmune Addison's disease (AD) is characterised by the presence of autoantibodies (Abs) to steroid 21-hydroxylase (cytochrome P450 C21) in patients' sera (1–3). P450 C21 is a heme containing membrane-bound enzyme that catalyses the conversion of progesterone and 17α-hydroxyprogesterone into deoxycorticosterone (DOC) and 11-deoxycortisol respectively (4). Hydroxylation reactions require a molecular oxygen and electron transfers from nicotinamide adenine dinucleotide phosphate reduced (NADPH) via NADPH-cytochrome P450 reductase (CPR) (5). The heme binding site as well as the site for interaction with the CPR are situated in the C-terminal region of the P450 C21 molecule (6, 7). The correct tertiary structure of P450 C21 is important for P450 C21 enzyme activity (8, 9).

Previous studies have shown that P450 C21 Abs in sera from patients with AD have an inhibiting effect on P450 C21 enzyme activity *in vitro* (10) although this effect does not appear to be evident *in vivo* (11). P450 C21 Abs react with conformational epitopes located mostly in the central and the C-terminal part of the P450 C21 molecule (amino acids (aa) 280–494) and a close relationship between P450 C21 Ab-binding sites and sites important for P450 C21 enzyme activity has been reported (3, 12–15).

We have studied two possible mechanisms for the inhibition of P450 C21 activity by P450 C21 Abs: (a) conformational changes in the P450 C21 molecule induced by the Ab binding and (b) the effects of the Abs binding to P450 C21 on the electron transfer from the CPR to P450 C21.

Materials and methods

P450 C21 Ab preparations

Sera from ten patients (P1–P10; Table 1) with autoimmune AD and P450 C21 Ab levels ranging from 16 units/ml to 2664 units/ml were used in the study.

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Table 1 Characteristics of IgG samples used in the study and the effect on the fast phase reduction of P450 C21.

IgG preparations	Diagnosis	P450 C21 Ab (unit/ml) Serum	P450 C21 Ab (unit/ml) 1 mg/ml IgG	Inhibition of [^{125}I] P450 C21 binding (%) by mouse MAb Fabs mixture[a]	P450 C21 reduced in fast phase (% reduced with dithionite; means±S.D.; n=2)
P1	APSII	226	76.7	87	(−1.7)±1.2
P2	APSII	2664	114.1	81	1.3±1.6
P3	AD	1852	1029.3	93	3.0±3.1
P4	AD	35.6	30	92	2.7±0
P5	APSII	50.2	20.5	93	4.2±1.2
P6	APSII	196.6	78.9	81	4.3±0.3
P7	APSII	736.7	472.4	90	4.2±0.6
P8	AD	16.1	15.7	89	4.2±1.3
P9	AD	36.4	33.9	84	7.8±0.6
P10	AD	26.1	13.4	83	11±1.4
HBD1	HBD	<1.0	<1.0	NA	12.5±2.1
HBD2	HBD	<1.0	<1.0	NA	9.6±2.5
HBD3	HBD	<1.0	<1.0	NA	11.3±1.1

[a] Inhibition of P450 C21 Ab binding to ^{125}I-labelled P450 C21 was analysed using a mixture of Fabs prepared from three mouse monoclonal antibodies (MAbs 1, 3 and 5) reactive with different epitopes on P450 C21 (17). MAb 1 reacted with aa 391–405, MAb 3 reacted with aa 406–411 and MAb 5 reacted with aa 335–339. The experiments were carried out as described previously (17). Fabs, antigen binding fragments of IgG; Na, not applicable (these IgGs did not bind to [^{125}I]P450 C21).

Five out of ten sera were from patients with isolated AD and five out of ten were from patients with autoimmune polyglandular syndrome (APS) type II (3). P450 C21 Abs were measured using an assay based on ^{125}I-labelled recombinant P450 C21 (16) (kit from RSR Ltd, Cardiff, UK). IgG was isolated from the sera by chromatography on ProsepA (Millipore UK Ltd, Watford, Herts, UK) according to the manufacturer's instructions, dialysed against phosphate-buffered saline (PBS; 8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, 2.7 mmol/l KCl and 137 mmol/l NaCl, pH 7.3) and stored in aliquots at −70 °C. The IgG concentration was calculated on the basis of absorbance (Abs) at 280 nm of 1.0 = 0.70 mg/ml. In addition, IgGs were isolated from three P450 C21 Ab-negative healthy blood donor (HBD1-3) sera. The ability of IgGs isolated from AD sera and HBD sera to bind ^{125}I-labelled P450 C21 was tested in the same way as serum P450 C21 Abs (16).

Patients gave informed consent for the study and HBD sera were purchased from Golden West Biologicals (Vista, CA, USA).

Recombinant human P450 C21 preparations

Full length recombinant human P450 C21was expressed in *Saccharomyces cerevisiae* as described previously (16, 18). Yeast transformed with P450 C21 cDNA was grown, harvested and broken as described previously (16, 18). The broken cells were centrifuged at 17 000 ***g*** for 30 min at 4 °C, and the supernatant was separated and then centrifuged at 105 000 ***g*** for 1 h at 4 °C. The microsomal pellet thus obtained was suspended in PBS followed by resedimentation at 105 000 ***g*** for 1 h at 4 °C and this cycle was repeated. The final pellet was resuspended in PBS (protein concentration of 10 mg/ml as determined by the Bradford assay) (19) and stored in aliquots at −70 °C.

Determination of P450 C21 enzyme activity

P450 C21 enzyme activity in the microsomal preparations was measured in terms of conversion of [^{3}H]progesterone to [^{3}H]DOC as described previously (5, 10, 20). IgGs from AD patients and HBDs were incubated with the microsomes in order to study the effect of IgGs on P450 C21 enzyme activity. Briefly, yeast microsomes containing recombinant P450 C21 (0.5 μg protein/1 μl PBS) were incubated with IgG preparations (25 μg, 50 μg or 100 μg) in 50 μl PBS at 0 °C for 16 h. The CPR (9.4 pmol/0.7 μl in 50 mmol/l potassium phosphate buffer, pH 7.2 containing 20% (v/v) glycerol, 0.1 mmol/l EDTA and 0.1 mmol/l dithiothreitol), purified from bovine liver microsomes as described previously (20) was then added to the microsome–IgG mixture. After an incubation at 0 °C for 1 h, 5 nmol progesterone with 0.2 μCi [^{3}H]progesterone (Perkin Elmer Life Sciences, Boston, MA, USA) in 500 μl of 50 mmol/l potassium phosphate buffer (pH = 7.2) containing 0.1 mmol/l EDTA was added and incubated at 37 °C for 1 min. The hydroxylation reaction was initiated by the addition of 10 μl of 10 mmol/l NADPH (Roche Diagnostics, Lewes, East Sussex, UK). After 40 min of incubation at 37 °C, the reaction was terminated by vigorous shaking with 1 ml chloroform containing 1.5 nCi [^{14}C]DOC as an internal standard. [^{14}C]DOC was prepared enzymatically from [^{14}C]progesterone (Perkin Elmer Life Sciences) and purified by HPLC. [^{3}H]DOC, the metabolite of [^{3}H]progesterone, was separated by HPLC (JASCO PU-980 intelligent HPLC pump, Metrotech Services Ltd, Cork, Ireland; TSK UV-8 model-II, TSK TOYO, Soda, Japan; Tosoh AS-8000, TOSOH, Tokyo, Japan; Gilson 202, Gilson Inc., Middleton, IW, USA) using a normal phase silica-gel column (4.6 × 150 mm; Cosmosil 5SL; Nacalai Tesque, Kyoto, Japan) with hexane, isopropyl alcohol and acetic acid (in the ratio of 93:7:1 respectively) at a flow rate of 0.8 ml/min (20).

Measurement of CO difference spectra of P450 C21 preparations

Aliquots of 10 μg yeast microsomes (in 20 μl PBS) were preincubated with 420 μg IgG preparations (in 210 μl PBS) at 0 °C overnight and mixed with 480 μl reaction buffer (50 mmol/l potassium phosphate buffer, pH = 7.2, containing 0.1 mmol/l EDTA, 10 μmol/l progesterone and 50 mmol/l glucose). A few grains of dithionite (Nacalai Tesque) were added to the solution, mixed and incubated at room temperature for 5 min. The baseline spectrum of the reaction mixture was recorded (Beckman DU640s spectrophotometer, Beckman Coulter Inc., Fullerton, CA, USA) from 500 nm to 400 nm. Subsequently, CO gas was bubbled gently through the dithionite-reduced solution for a few seconds and the dithionite-CO difference spectra recorded at 25 °C (21). The difference spectra were obtained by subtraction of the baseline absorbance from the absorbance after dithionite reduction and CO bubbling.

Assessment of electron transfer from CPR to P450 C21

Aliquots containing 7.5 μg yeast microsome protein in 15 μl PBS were incubated with 280 μg IgG preparations (in 140 μl PBS) at 0 °C overnight. Then, 153 pmol CPR (9 μl) was added and incubated for 1 h at 0 °C. Reaction buffer (320 μl; see above) was degassed and bubbled with CO gas for a few seconds. This step was repeated three times. The mixture of the CPR, yeast microsomes and the IgG was then injected into the CO-saturated reaction buffer. The solution was further degassed and flushed with CO gas at least three times and the residual oxygen was removed by an oxygen scavenging system composed of 2 units glucose oxidase (from *Aspergillus niger*; Sigma-Aldrich Company Ltd, Poole, UK) and 60 units catalase (from bovine liver; Sigma-Aldrich Company Ltd, Poole, UK). After incubation at 25 °C for 5 min, the reaction was initiated by mixing the reaction mixture (502 μl) with 6 μl of 10 mmol/l NADPH in distilled water and the difference in the spectra of the reaction mixture before and after adding NADPH was recorded at 25 °C. The spectra were measured at 1, 2, 3, 5, 10, 20, 30, 40 and 55 min after NADPH addition. Subsequently, 2 μl saturated dithionite solution was added to the sample and the difference spectrum was recorded (complete reduction of P450 C21).

Statistical analysis

Statistical analyses of differences between the effect of patient and HBD IgGs on P450 C21 enzyme activity and the effect on electron transfer from CPR to P450 C21 were carried out using ANOVA in Origin version 6 (Rockware Inc., Golden, CO, USA). Values are given as means±S.D.

Results

The P450 C21 Ab activities of IgG preparations (P1–P10; Table 1) ranged from 13.4 to 1029 units/ml for 1 mg/ml solution of IgG when assessed using the [^{125}I]P450 C21 assay (units are the kit calibrators). P450 C21 Ab activity was undetectable in IgG preparations from HBD sera.

P450 C21 enzyme activity in the microsomal preparation obtained from yeast transformed with P450 C21 cDNA, expressed as the ability to convert progesterone to DOC, was 1.22±0.25 ($n = 12$) nmol DOC produced/40 min per 0.5 μg microsomal protein. In contrast, DOC formation was undetectable (less than 0.02 nmol) in the absence of NADPH.

The effect of P450 C21 Ab on P450 C21 enzyme activity was expressed as the percentage of the activity remaining in the presence of patient IgG relative to the P450 C21 enzyme activity in the presence of HBD IgG. In the presence of 100 μg IgG from P1, P2 or P3, the remaining P450 C21 enzyme activity was 10±0.9%, 5±1.1% and 4.5±1.3% (closely agreeing duplicates) relative to the P450 C21 enzyme activity in the presence of HBD2 IgG respectively (Fig. 1). The differences in P450 C21 enzyme activity remaining in the presence of P1, P2 or P3 IgGs compared with HBD IgG were statistically significant ($P < 0.001$ in each case). In the case of experiments carried out with different concentrations of P3 IgG (25 μg, 50 μg or 100 μg), the remaining P450 C21 enzyme activity was 50±3%,

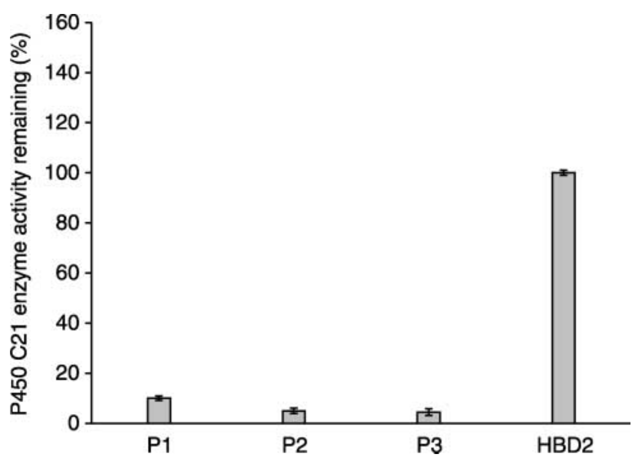

Figure 1 P450 C21 enzyme activity remaining in the presence of 100 μg IgGs from AD patients (P1, P2 or P3) relative to P450 C21 enzyme activity in the presence of 100 μg HBD2 IgG. Results shown are means of closely agreeing duplicates±S.D. The differences in P450 C21 enzyme activity remaining in the presence of P1, P2 or P3 IgGs compared with the activity remaining in the presence of HBD IgG were statistically significant ($P < 0.001$ in each case).

30±2.5% and 4.5±1.3% (closely agreeing duplicates), relative to the P450 C21 enzyme activity in the presence of the same concentrations of HBD2 IgG. P4–P10 IgGs (Table 1) were shown to have en effect on P450 C21 enzyme activity (2–63% of enzyme activity remaining) in our previous study (10).

The conformation around the heme in the P450 molecule can be deduced from the dithionite-reduced CO difference spectra (21). The native form of P450 shows a peak at 450 nm in the difference spectra while the denatured form shows a peak at 420 nm. An analysis of dithionite-reduced CO difference spectra of P450 C21 in yeast microsomes in the presence of patient P2 and HBD3 are shown in Fig. 2. The difference spectra showed a peak at 450 nm in the presence of P2 or HBD3 IgGs (Fig. 2a and b respectively). The value of difference of absorbance at 450 nm minus absorbance at 490 nm (ΔOD) in the presence of HBD IgG was 0.005±0.0005 ($n = 2$) and was comparable with 0.0056±0.0005 (n=2) in the presence of P2 IgG. There was no peak evident at 420 nm in the difference spectra of the P450 C21 preparations incubated with P2 IgG or HBD3 IgG (Fig. 2). Similar results were obtained when P1, P3–P10 and HBD1 and HBD2 IgGs were tested (data not shown).

Electron transfer from the CPR to P450 C21 heme can be detected by an increase of absorbance at 450 nm which is caused by immediate binding of CO to the reduced heme in P450 C21 (22). The increase of absorbance at 450 nm in the difference spectra of P450 C21 in the presence of HBD3 IgG after incubation with NADPH is shown in Fig. 3a. The absorbance at 450 nm increased rapidly to ΔOD Abs=0.0016 in 1 min after addition of NADPH and then increased slowly to about ΔOD=0.0055 at 55 min. In the case of P450 C21 preparations incubated with P2 IgG there was no peak at 450 nm at 1 min after incubation with NADPH (Fig. 3b). However, an increase of absorbance at 450 nm was observed over 2–55 min (max ΔOD of 0.0048) after addition of NADPH (Fig. 3b).

The plot of an increase of absorbance at 450 nm over 55 min after addition of NADPH in the presence of HBD3 IgG or P2 IgG is shown in Fig. 4. As illustrated, the contribution of slow phase reduction within 1 min must be considered in calculating the fast phase reduction and consequently the true fast phase

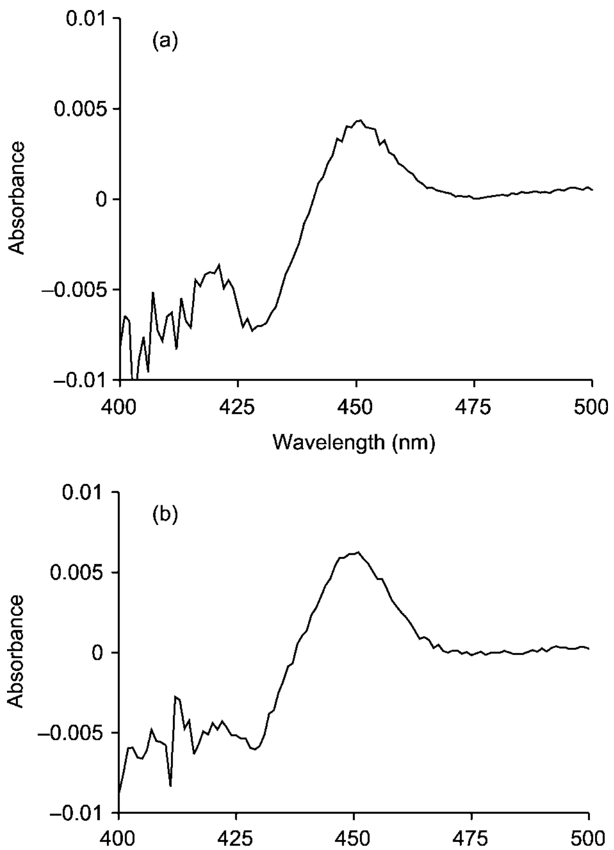

Figure 2 Dithionate-reduced CO difference spectra of P450 C21 in the presence of (a) HBD3 IgG and (b) AD patient P2 IgG. The difference spectra show a peak at 450 nm but not at 420 nm in the presence of P2 or HBD3 IgGs.

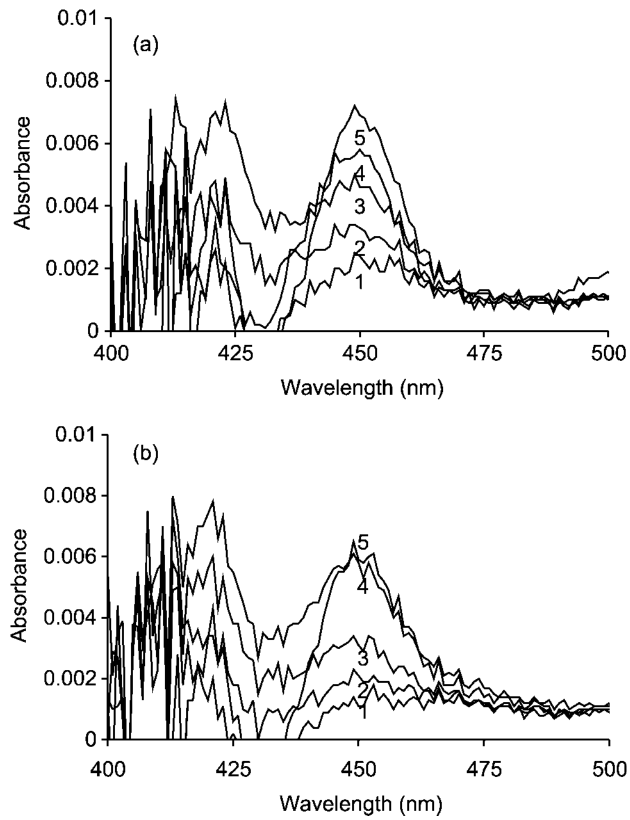

Figure 3 CO difference spectra of P450 C21 in the presence of (a) HBD3 IgG and (b) AD patient P2 IgG. Absorbances represented by curves 1 to 4 were recorded after 1, 5, 20 and 55 min respectively after incubation with NADPH. Absorbances shown by curve 5 represent the spectrum after addition of dithionate (complete reduction of P450 C21).

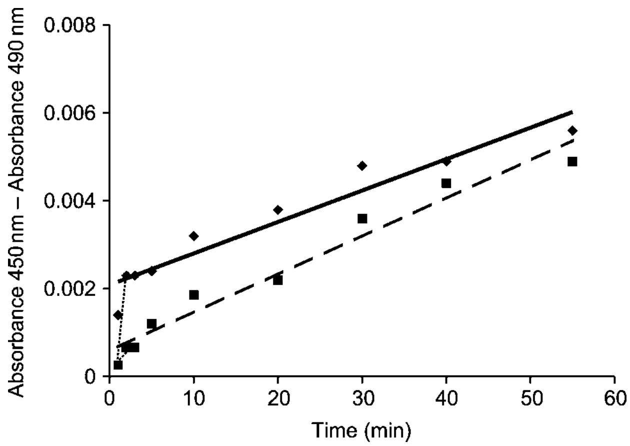

Figure 4 Plot of an increase in absorbance of P450 C21 at 450 nm over 55 min after addition of NADPH in the presence of HBD3 IgG (◆) or P2 IgG (■). The dotted line represents fast phase reduction within 1 min (see text for details).

reduction within 1 min was obtained by subtracting the effect of slow phase reduction from the observed ΔOD at 1 min, i.e. [observed ΔOD in 1 min] − [(slope in the slow phase (ΔOD/min) × 1 min)] (Fig. 4). In the case of incubation of P450 C21 with HBD3 IgG, the value of true fast phase reduction was 0.0005 compared with 0.0001 in the case of incubation with P2 IgG.

The proportion of P450 C21 reduced in a rapid phase in the presence of P1–P10 or HBD1–3 IgGs expressed as a percentage of P450 C21 reduced with dithionite: [(ΔOD in 1 min) − (slope in the slow phase (ΔOD/min)) × 1 min)]/[(ΔOD after dithionite reduction]] is shown in Table 1.

There was a statistically significant difference in the percentage of P450 C21 reduced in the rapid phase in the case of incubation with nine out of ten IgGs from AD patients (P1–P9) compared with HBD IgG ($P < 0.05$ in each case). However, in the case of P10 IgG, the percentage of P450 C21 reduced in the rapid phase was not statistically different compared with HBD IgG ($P = 0.86$).

Discussion

Incubation of P450 C21 preparations with P1–P3 IgGs resulted in an inhibition of P450 C21 enzyme activity (Fig. 1) and this is in agreement with the previously reported effect of P4–P10 IgGs (Table 1) (10). Furthermore, the degree of the inhibiting effect of P1–P3 IgGs on P450 C21 enzyme activity was similar (the remaining P450 C21 enzyme activity was 10, 5 and 4.5% in the case of incubation with P1, P2 and P3 IgGs containing 76.7 units/mg, 114.1 units/mg and 1029 units/mg P450 C21 Ab respectively (Table 1)). This is consistent with our previous observations that the degree of inhibition of P450 C21 enzyme activity was not related to the level of P450 C21 Abs in the IgG preparations (10). The aim of this study was to investigate the possible mechanism by which P450 C21 Abs inhibit P450 C21 enzyme activity. Correct conformational folding of the P450 C21 molecule and availability of an efficient system for electron transfer are assumed to be essential for P450 C21 enzyme activity (8, 9, 13–15, 22). Thus, there are at least two possible mechanisms by which P450 C21 Abs may inhibit P450 C21 enzyme activity: (a) changes in the conformation of the P450 C21 molecule brought about by binding of P450 C21 Abs and/or (b) interference in the electron transfer from the CPR to the P450 C21 heme as a result of P450 C21 Ab binding to P450 C21.

Analysis of dithionite-reduced CO difference spectra is a useful method to assess the conformation around the heme groups in P450 cytochrome proteins (21). The difference spectra of P450 proteins in their native conformation show a peak at 450 nm whereas in the case of denatured P450 proteins the highest absorbance is observed at 420 nm (21). We have studied the difference spectra of P450 C21 following incubation with IgGs isolated from P450 C21 Ab-positive AD patients or HBD. The difference spectra following incubation of P450 C21 with either P450 C21 Ab-positive or P450 C21 Ab-negative IgGs showed a peak at 450 nm. Furthermore, the peak height was similar after incubation with both types of IgGs (Fig. 2). There was no peak at 420 nm indicating that the conformation of P450 C21 around the heme molecule had not changed upon binding of P450 C21 Abs.

Electron transfer from NADPH-P450 reductase to P450 C21 can be measured by monitoring absorbance at 450 nm in the presence of CO (22). In the reaction, as soon as the P450 heme accepts an electron, CO binds to the heme and this is reflected by an increase of absorbtion at 450 nm. The physiological rate of electron transfer is fast; transfer of one electron from the CPR to P450 C21 in the P450 C21 enzyme activity reaction is estimated to take only a few seconds (22). Consequently, changes in the fast phase (within 1 min) of difference spectra at 450 nm are indicative of electron transfer related to P450 C21 enzyme activity (22). Figure 3a shows a rapid increase of a peak at 450 nm in the difference spectra within 1 min (curve 1) after addition of NADPH in the reaction with P450 C21 preparations preincubated with IgG from an HBD. However, there was no change of a peak at 450 nm (within 1 min after addition of NADPH) in the difference spectra in the case of the experiment carried out using P450 C21 preparations preincubated with P2 IgG (Fig. 3b, curve 1). These experiments allowed an assessment of the rate of the fast phase reduction of P450 C21 that was shown to be slower in the presence of nine out of ten patient IgGs compared with HBD IgG (Fig. 4 and Table 1). The degree of the effect of P450 C21 Ab IgGs on the fast phase reduction of P450 C21 did not appear to be related to the levels of P450 C21 Ab in the IgG preparations or the form of AD (isolated AD or APS type II)

(Table 1). The complexity of the experiments and the limited availability of patient IgGs did not allow us to extend the study with larger numbers of samples or samples from patients with APS type I or patients who were positive for P450 C21 Abs but had normal adrenal function. However, previous studies have shown that the binding characteristics of P450 C21 Ab in sera from patients with different forms of AD and P450 C21 Ab-positive patients without overt adrenal failure are similar and likely to recognise closely related epitopes on P450 C21 (3, 15, 17, 23). Furthermore, binding of all IgGs used in our study (P1–P10) to ^{125}I-labelled P450 C21 was inhibited by a mixture of the three mouse MAb Fabs reactive with the epitopes within the C-terminal part of the P450 C21 molecule (aa 335–339, aa 391–405 and aa 406–411) (Table 1) (17). Mouse MAb Fabs inhibited most of the P450 C21 Ab activity in P1–P10 IgGs (81–93% inhibition; Table 1) indicating that the epitopes recognised by these Abs were closely related and located predominantly in the C-terminal part of P450 C21.

In our study, P10 IgG (only one out of ten patient IgGs studied) did not show a statistically significant effect on the fast phase P450 C21 reduction (Table 1). This IgG had a relatively small effect on P450 C21 enzyme activity (63% enzyme activity remaining in the presence of P10) (10) although the levels of P450 C21 Ab in this preparation were comparable with the levels in other preparations (Table 1).

The rate of slow phase electron transfer (reduction of P450 C21) was similar in the reactions with P450 C21 preparations in the presence of P450 C21 Abs or normal IgGs (Fig. 3a, curves 2–4; Fig. 3b, curves 2–4 and Fig. 4). Two step reduction of P450 cytochrome proteins in microsomal preparations or in the reconstituted systems (the mixture of purified P450 and the CPR) has been reported previously (22). However, it has been suggested that the slow rate of electron transfer is too slow to be effective for physiological catalytic activity of P450 C21 (22).

In this study we have not been able to detect conformational changes around the heme binding site in the P450 C21 molecule following binding of P450 C21 Abs. It is unlikely therefore that P450 C21 Abs inhibit P450 C21 enzyme activity through the conformational changes of P450 C21 brought about by P450 C21 Ab binding. However, our experiments do indicate that P450 C21 Abs in AD sera inhibit the fast phase of electron transfer from the CPR to P450 C21. Consequently, inhibition of electron transfer seems the most likely mechanism by which P450 C21 Abs inhibit P450 C21 enzyme activity. Alternatively, P450 C21 Ab binding may result in subtle conformational changes within the P450 C21 molecule that were not detected in the dithionite-reduced CO difference spectra experiments that we have carried out. It is not clear at present how P450 C21 Abs inhibit the electron transfer. One of the ways could be that P450 C21 Abs inhibit the interaction between the CPR and the P450 C21 molecule. For example, P450 C21 Ab-binding sites may be closely related to the CPR interaction site on P450 C21. Previous studies have shown the relationship between P450 C21 Ab-binding sites and sites important for P450 C21 enzyme activity (3, 10, 12–15). Furthermore, the P450 C21 Abs used in this study were directed predominantly to epitopes within the C-terminal part of P450 C21 known to be involved in interaction with the CPR (6, 7, 9, 24). However, the detailed location of P450 C21 epitopes involved in P450 C21 Ab binding has not been identified as yet (3, 12–15, 17, 23). It may well be that the lack of a clear effect on the fast phase reduction of P450 C21 by P10 in our study is related to the subtle differences in the binding sites for different P450 C21 Abs. Although our studies provide an insight into the mechanism of the inhibiting effect of P450 C21 Abs on P450 C21 enzyme activity *in vitro*, the significance of this inhibition for the pathogenesis of AD *in vivo* needs to be studied further (3). Also, more detailed analysis of the epitopes involved in P450 C21 Ab binding and their relationship to the CPR-binding site should be helpful in elucidating the mechanism by which P450 C21 Abs inhibit the fast phase electron transfer between the CPR and P450 C21.

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