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Antiethinyloestradiol antibody activities in oral contraceptive users

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SUMMARY

Oral contraceptives (OC) can induce in some women, serum immune complexes precipitated in 25% saturated $(NH_4)_2SO_4$, especially in the case of a thrombotic complication. In this work, the presence of antiethinyloestradiol (anti-EE) antibodies in the complexes was investigated. Binding of ethinyloestradiol-^{3}H (EE-^{3}H), either by $(NH_4)_2SO_4$ precipitate directly, or in equilibrium dialysis experiments with the anti-EE gamma-globulin isolated through a purification-activation method combining dilution chromatography and affinity chromatography, was measured. The purified proteins were identified by immunoprecipitation and isoelectrophoresis.

Twelve sera from OC users and three from women who had never used them were tested. Eleven of the twelve users had serum immune complexes. Normal human gamma-globulin pools and gamma-globulins prepared from a rabbit immunized with EE, were used as negative and positive controls.

Results demonstrated that serum complexes precipitated in 25% saturated ammonium sulphate contained EE-^{3}H binding gamma-globulins. For seven out of eleven cases, the association constant K_a was calculated and varied from 4×10^5 M^{-1} to $2{\cdot}6 \times 10^7$ M^{-1}, and the valence from 1·4 to 2, a result consistent with an IgG antibody activity.

These anti-EE antibodies were found in the serum complexes from women on OC with or without thrombosis. However their amount seemed to be greater in the cases with thrombosis.

In the serum of women who had never used the pill, in the only OC user who had no serum complexes in 25% saturated ammonium sulphate, and in the pool of normal human gamma-globulins, no anti-EE activity was detected.

INTRODUCTION

Immune complexes, precipitated in 25% saturated ammonium sulphate and containing a monoclonal IgG λ with antiethinyloestradiol activity, were found in the serum of a 36-year-old woman who had experienced pulmonary artery thrombosis during oral contraception. This first case report (Beaumont & Lemort, 1976) suggested: (1) that synthetic oestrogens may stimulate the production of specific antibodies, even through oral administration; (2) that the antibodies, or the complexes they form with hormones *in vivo*, may have pathological and, particularly, thrombotic consequences.

To test this hypothesis, the possibility of a correlation between oral contraceptives, antibody formation and thrombosis, had to be investigated in other cases.

First the correlation was statistically demonstrated (Beaumont *et al.*, 1978): circulating immune complexes, precipitated in 25% saturated ammonium sulphate, were found in nearly 30% of oral contraceptive users, in more than 90% of those with a thrombotic complication, and not in untreated controls.

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In this work, the nature of the correlation was investigated: the immunoglobulins purified from the circulating complexes in the above groups of women, are shown to be antiethinyloestradiol antibodies.

MATERIALS AND METHODS

Serum samples were obtained from three groups of women: Group 1—three women, aged 17–46 years, who had never taken oral contraceptives (OC), nor hormonal therapy. Group 2—6 women, aged 21–49 years taking OC, with serum immune complexes precipitated in 25% saturated ammonium sulphate. Group 3—6 women, aged 21–38 years, with arterial thrombosis in the course of OC (Table 1). In this group, is the only case of monoclonal gammapathy (IgG λ MAI) case 1.

TABLE 1

		Oral contraception		
Cases	Age (years)	Duration of use (months)	EE dose/day (μg)	Thrombotic complications
1	38	29	50	Pulmonary artery thrombosis
2	36	6	50	Popliteal artery thrombosis
3	38	19	50	Femoro-popliteal thrombosis
4	23	36	50	Pulmonary artery thrombosis
5	36	84	50	Iliac artery thrombosis
6	21	18	50	Thrombosis of the basilar trunc

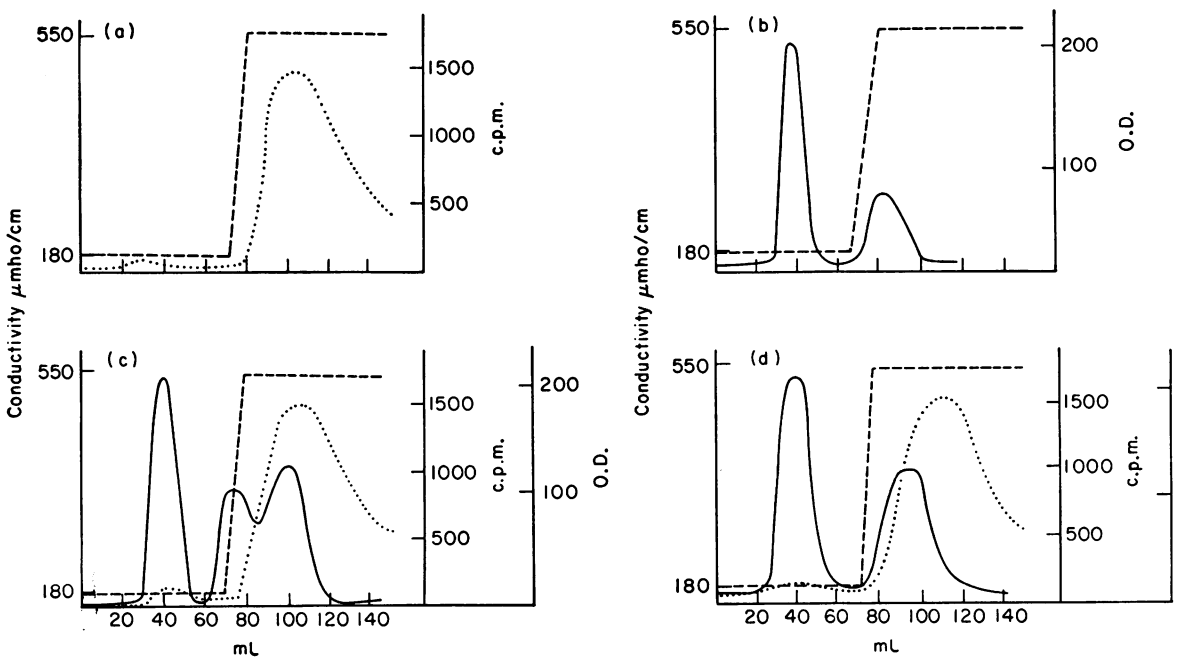

FIG. 1. Intervent dilution chromatography on 40×2 cm columns of a DEAE Sephadex A 25/Sephadex G 25 1/1 v/v mixture, prepared in 0·015 M barbital acetate, 0·05 M NaCl, pH 9·4 buffer, and eluted with a 0·015 M barbital acetate, 0·5 M NaCl, pH 9·4 buffer. The elution patterns show the changes in molarity (- - - -) scintillation counts (. . . .), and optical density at 280 μm (———), when EE-^{3}H is chromatographied alone (a) and mixed with rabbit anti-EE-^{3}H immunoglobulins prepared at 30% $(NH_4)_2SO_4$ (c) or with the immune complexes prepared with 25% $(NH_4)_2SO_4$ in one OC user (d). The elution pattern obtained with purified human IgG is given in (b). The proteins found in the first peaks eluted before the molarity front are mainly IgG.

Immunoglobulin activities. (1) *Detection and estimation of the antiethinyloestradiol antibody (anti-EE ab) activity*: in the case of IgGλMAI, the immunoglobulin (Ig) in the serum was almost entirely complexed and inactive. Estimation of the anti-EE activity needed separation and purification of the Ig from the blocking antigens.

This method of 'purification-activation' was applied in the present work, together with a direct simplified, but less precise method. In both methods, the activity was estimated from the binding of tritiated ethinyloestradiol (EE-^{3}H).

The 'purification-activation' method was performed as follows: (a) Precipitation, from undiluted serum, of circulating immune complexes in 25% saturated $SO_4(NH_4)^2$, and washing with the same solution.

The precipitate was essentially made of immunoglobulins, especially IgG, small quantities of IgA and IgM and traces of albumin.

The precipitate was abundant in all the sera of group 2, and 5 out of 6 in group 3, small in 1 of group 3 and in the group 1. In the latter, a precipitation in 30% $SO_4(NH_4)^2$ was also performed, and the precipitate treated in the same way.

(b) An intervent dilution chromatography, according to the method of Kierkegaard & Coe Agee (1973), was performed on a 40 × 2 cm column of a DEAE Sephadex A-25/Sephadex G-25 1:1 mixture, conditioned in a 0·015 M barbital acetate, 0·05 M NaCl buffer pH 9·4.

Complexes issued from (a) and dissolved in 1 ml of 0·015 M barbital acetate, 0·5 M NaCl pH 9·4 buffer, were layered on the column and eluted with the same buffer. Most of the Igs are eluted in the first peak of molecules which are excluded from Sephadex G-25 and not retained on Sephadex A-25 at a 0·05 M molarity. Under these conditions, steroid hormones, namely ethinyloestradiol, are retained and partly eluted later with the 0·5 M NaCl molarity front.

(c) An affinity chromatography performed with an EE/AH Sepharose gel obtained by coupling azoethinyloestradiol prepared by diazotation, to a AH-Sepharose gel.

The Igs issued from (b) were mixed with the EE/AH-Sepharose gel by gentle rotation, in the ratio of 2 mg protein in 1 ml 0·015 M veronal acetate, 0·5 M NaCl buffer pH 9·4 for 1 g of gel. After a 1 hr rotation, the gel was dialysed against the above buffer, but without NaCl, then deposited on a small column and washed with 1 litre of the same buffer, to get rid of the non-coupled molecules. Then the anti-EE Ig was finally eluted with 0·015 M barbital buffer, pH 9·4 after addition of 0·5 M NaCl.

The radioactivity in the external and internal chambers was measured after a 24-hr dialysis at +4°C in a scintillation counter Nuclear Chicago. Association constant (K_a) and valence were estimated on a Scatchard diagram (Scatchard, 1949), each point of the diagram representing the mean of the measurements made for three aliquot samples of the studied protein.

The simplified direct method. This consists in measuring the direct binding of EE-^{3}H to the antibodies of the serum complexes.

Proteins precipitated in 25% saturated ammonium sulphate were dialysed against 0·1 M phosphate buffer pH 7·3. Isotopic dilutions of 50 μl EE-^{3}H and increasing quantities of unlabelled EE from 1 to 10 μg were prepared and added to 500 μg of the above dialysed proteins. After a 40-hr incubation at +4°C, the Igs were precipitated in 15% polyethyleneglycol (PEG), centrifuged washed in 15% PEG and the new precipitate was dissolved in 0·1 M phosphate buffer pH 7·5. The radioactivity was measured in a scintillation counter, and the binding of EE-^{3}H expressed in c.p.m. for 500 μg of the studied proteins, compared to the binding obtained with 500 μg of a standard Ig mixture.

(2) *Specificity of the reactions*: Controls were included in both methods: (a) normal sera, human and bovine albumins, and purified Igs were tested as possible ligands; (b) negative controls (normal serum and normal Ig) and 1 positive control (rabbit anti-EE antiserum) were used in each experiment. The anti-EE antiserum was prepared from a rabbit injected with EE coupled to thyroglobulin.

The antihormone specificity. This was studied: (a) in equilibrium dialysis experiments by the estimation of the association constant (K_a) of 17-beta-oestradiol-^{3}H (C.E.A.) and EE-^{3}H (Roussel). Also: (b) in equilibrium dialysis and with the direct method by measuring the inhibition of EE-^{3}H binding obtained with different hormones: ethinyloestradiol (Sigma), 17-beta-oestradiol (Sigma), progesterone (Vister, Italy), oestradiol (Vister), testosterone (Sigma), hydrocortisone (Vister) and in some experiments: diethylstilbestrol (Calbiochem), hexestrol (Sigma), didrogesterone (Sigma), oestrone (Vister), androsterone (Sigma), and cortisol (Sigma).

Identification of the proteins. The proteins issued from the purification-activation method were identified by polyacrylamide gel isolectrofocussing (Vesterberg, 1972), immunoprecipitation in double diffusion (Ouchterlony, 1958), and immunoelectrophoretic (Grabar & Williams, 1953) experiments with appropriate antisera (Hyland Laboratories).

RESULTS

Antiethinyloestradiol activity

It was possible to demonstrate the antiethinyloestradiol activity in a serum by both the direct and indirect methods, but the purification-activation method only, followed by the estimation of EE-^{3}H binding in equilibrium dialysis, allowed the determination of K_a and valency.

Direct method (Table 2). Using pure EE-^{3}H without any dilution with cold EE, showed that the binding of EE-^{3}H by the proteins precipitated in 25% saturated ammonium sulphate (or in 30% when there was no precipitation at 25%) was greater in groups 2 and 3 than in group 1. In the latter group, the

TABLE 2. Binding of ethinyloestradiol-^{3}H by the serum proteins. (Here EE-^{3}H is not diluted with cold EE and binding is measured by the direct methods.)

Groups	Case no.	Saturation in $SO_4(NH_4)z$ at which the proteins were prepared		EE-^{3}H binding by 500 μg proteins (c.p.m.)
		25%	30%	
	1	−	+	576
1	2	−	+	590
	3	−	+	580
	1	+		1060
	2	+		1240
2	3	+		1355
	4	+		600
	5	+		925
	6	+		630
	1	+		2412
	2	+		1160
3	3	+		2500
	4	+		1030
	5	—	+	670
	6	+		2300
Normal gamma-globulins (control)			+	500
Gamma-globulins from anti-EE rabbit			+	20,000

binding was similar to that obtained with the Ig used as control (about 500 c.p.m. for 500 μg protein). The binding obtained with the rabbit anti-EE antibodies in these conditions was 20,000 c.p.m. for 500 μg proteins precipitated at 30% ammonium sulphate.

Three cases reacted differently: case 4 and 6 in group 2, in which proteins precipitated at 25% and did not bind EE-^{3}H, and case 5 in group 3, in which serum could only be precipitated at 30% and did not bind EE-^{3}H.

Finally, the increased binding of EE-^{3}H by the sera of groups 2 and 3 and the rabbit anti-EE antiserum could be inhibited by addition of unlabelled EE.

Purification-activation method. The binding of EE-^{3}H measured in equilibrium dialysis experiments, was different in the three groups (Table 3).

In group 1 (controls), the precipitation in 25% saturated ammonium sulphate yielded only small quantities of proteins. These proteins were not retained on the EE/AH-Sepharose column, and at the end of the purification procedure, there were only traces of proteins which did not bind EE-^{3}H.

In group 2 (women on oral contraceptives without thrombosis), all six sera precipitated heavily in 25% saturated ammonium sulphate and, after an intervent dilution chromatography, most of the proteins in the precipitates were retained on the EE/AH-Sepharose gel; only part of them were eluted in 0·5 M NaCl, pH 9·4 and the yield was between 15 and 30% (Table 3). For the eluted proteins, it was possible to calculate in cases 1 and 5 a K_a of 6×10^5 M^{-1} and a valency near 2, using 150,000 Daltons as the molecular weight of Ig.

The K_a could not be estimated in the other cases because the points on the Scatchard diagram were not on a straight line, and there was not enough material for further studies.

In group 3 (arterial thrombosis during oral contraception), there was a heavy precipitation in 25%

TABLE 3. Binding of EE-^{3}H by purified and activated gamma-globulins (method 1) measured in equilibrium dialysis experiments

Groups	Case	Proteins issued from precipitation in $SO_4(NH_4)_2$ at 25% (Lowry) μg/ml serum	Proteins obtained by the purification-activation procedure procedure (Method 1) (μg/ml serum) 1st step: Intervent dilution chromatography	2nd step: Affinity chromatography*	Immunoglobulin identification	Binding of EE-^{3}H by the purified protein K_a	Estimated valency
	1	360	traces	traces	IgG	no fixation	
1	2	600	traces	traces	IgG	no fixation	
	3	300	traces	traces	IgG	no fixation	
	1	1800	1600	450	IgG $\lambda+\kappa$	6×10^5 M^{-1}	2
	2	2040	1800	225	IgG $\lambda+\kappa$	+‡	
2	3	1690	1125	150	IgG $\lambda+\kappa$	+‡	
	4	1860	800	100	IgG $\lambda+\kappa$	+‡	1·6
	5	1500	855	100	IgG $\lambda+\kappa$	6×10^5 M^{-1}	1·6
	6	1360	720	110	IgG $\lambda+\kappa$	+‡	
	1	10 205	5000	1000	IgG λ	$2{\cdot}6\times10^7$ M^{-1}	2
	2	1436	1000	200	IgG $\lambda+\kappa$	8×10^5 M^{-1}	2
3	3	3874	630	400	IgG $\lambda+\kappa$	1×10^6 M^{-1}	2
	4	1701	1230	270	IgG $\lambda+\kappa$	5×10^5 M^{-1}	1·4
	5	379	traces	traces	IgG $\lambda+\kappa$	no fixation	
	6	1600	600	160	IgG $\lambda+\kappa$	4×10^5 M^{-1}	1·6
Anti-EE rabbit γ-globulins						2×10^7 M^{-1}	2

* Proteins retained on the EE/AH-Sepharose column.
† Antibody prepared from whole serum by affinity chromatography on EE/AH-Sepharose.
‡ Evaluation of K_a impossible.

saturated ammonium sulphate for the sera of 5 out of the 6 cases. Most of the proteins were retained on the EE/AH-Sepharose gel, and the yield of proteins eluted was 20 to 60%. For the eluted Igs, K_a could be estimated from 4×10^5 M^{-1} to $2{\cdot}6\times10^7$ M^{-1}, with valencies near 2. For case 5, there was no precipitation at 25% and the precipitate at 30% yielded no active proteins.

Proteins prepared from the rabbit anti-EE antiserum according to the same protocol, were also tested in equilibrium dialysis and showed a K_a of 2×10^7 M^{-1} and a valency of 2.

Specificity of the anti-EE activity (Table 4)

In the case with a monoclonal gamma-globulin, the IgG λ MAI also bound 17-beta-E-^{3}H, but with a K_a of only $0{\cdot}4\times10^7$ M^{-1} as compared with $2{\cdot}7\times10^7$ M^{-1} for EE-^{3}H.

Experiments on inhibition of EE-^{3}H binding in all the cases with an anti-EE-^{3}H activity showed that maximum inhibition could be obtained with EE-^{3}H itself, and to a lesser extent with 17-beta-E-^{3}H. The inhibition with other natural or synthetic hormones was investigated in case 1 and 4 of group 3, serum 1 from group 2, and rabbit anti-EE antiserum. There was poor or no inhibition, except with estrone for the rabbit serum, and progesterone for serum MAI.

Identification of the protein

In the active fractions issued from the affinity chromatography, immunoelectrophoresis and immunoprecipitation revealed only Igs. IgG were always seen, alone and monoclonal (IgG λ) in one case (case 1 of group 3), polyclonal (IgG λ and κ) in all other cases.

TABLE 4

Tested sera	Inhibiting effect of different hormones on the EE-^{3}H binding of 500 μg proteins prepared by the direct method (% of the binding observed without hormone addition)			
	⩾80 p. 100	⩾50–< 80	⩾10–< 50	< 10 not significant
Anti-EE rabbit	EE (91) 17-β-E (90)		oestrone (38) testosterone (12)	progesterone didrogesterone androsterone oestriol corticosterone cortisone hexestrol diethylstilbestrol
Group 2 Case no. 1		EE (50)	17-β-E (35) didrogesterone (45) hexestriol (20) oestriol (22) oestrone (17) testosterone (15)	diethylstilbestrol progesterone androsterone cortisol corticosterone dihydrocortisone
Group 3 Case no. 1	EE (85)	17-β-E (63)	progesterone (49) didrogesterone (27) testosterone (30) oestriol (35) androsterone (20) dihydrocortisone (20) cortisol (17)	oestrone hydrocortisone diethylstilbestrol hexestrol
Group 3 Case no. 4		EE (58) 17-β-E (58)	didrogesterone (33) oestrone (50) cortisol (20) dihydrocortisone (20)	diethylstilbestrol testosterone progesterone androsterone hexestrol oestriol

In polyacrylamide gel immunoelectrophoresis, the reactive Igs appeared to be polyclonal, giving a microheterogeneous spectrum in the Ig zone, except for the monoclonal IgGλ MAI which gave only seven well characterized bands. In some cases, albumin traces were seen in the preparations on isoelectrophoresis. Following an affinity chromatography on antialbumin gel, albumin was retained and the eluted Igs still had anti-EE activity.

DISCUSSION

This work confirms our first case report (Beaumont & Lemort, 1976) and shows that oral administration of synthetic hormones in OC may induce antiethinyloestradiol antibodies.

The association constants (K_a) of the Igs, from 10^5 M^{-1} to 10^7 M^{-1} are consistent with an antibody activity, but are lower than the K_a obtained with hyperimmunized rabbits which may reach 10^8 M^{-1}. However it is known that the antibodies from human autoimmune diseases often have an affinity similar to our cases (Seligmann & Brouet, 1973) and rabbits immunized in our laboratory with techniques known to give poor results, had K_a from 10^6 to 10^7 M^{-1}.

The reactions are specific of the ingested synthetic hormones. The finding of cross-reactions with 17-

TABLE 5.

Sera	Hormonal treatment	Thrombosis	Presence of precipitating complexes in SO_4 $(NH_4)_2$ at 25 p. 100 saturation	Protein	K_a	Inhibitors*	
						Majors	Minors
CAZ...	Oophorectomy Hexestrol implants	Thrombotic renal microangiopathy	+++	IgG $\lambda+\kappa$	6×10^5 M^{-1}	Hexestrol EE	Didrogesterone Progesterone DES
TRA...	Prostatic cancer Diethylstilbestrol	Humeral artery thrombosis	+++	IgG $\lambda+\kappa$	not measured	DES EE	Progesterone Testosterone Hexestrol
VAR...	Dysmenorrheas Didrogesterone	Multiple venous and artery thrombosis	+++	IgG $\lambda+\kappa$	4×10^5 M^{-1}	Didrogesterone 17-β-E	EE Progesterone

* In decreasing order EE: ethinylestradiol; DES: diethylstilbestrol; 17-β-E: 17-β-estradiol.

beta-oestradiol, the nearest natural hormone, is also significant. Valencies estimated on a Scatchard diagram, are found around 2, as may be expected for IgG.

These antibodies form serum complexes which precipitate in ammonium sulphate at a lower saturation than unbound Igs. An epidemiological study (Beaumont *et al.*, 1978) demonstrated that these particular complexes may be found in nearly 30% of women taking OC. A work in progress shows that they may be detected about 2 months after the beginning of oral contraception. Moreover they persist in serum even when the pill is discontinued, and this 'immunological scar' may be found after several years. As purified anti-EE antibodies are no longer precipitated in 25% saturated ammonium sulphate, the persistence of a precipitation after stopping EE suggests that the antibodies may still bind serum molecules other than EE but of similar structure.

The role of the anti-EE antibodies in the hormonal balance is not clear. It can be noticed: (1) that they do not inhibit the anti-fertility effects of the pill, as they were found in 30% of women on oral contraceptives; (2) that they allow a return to a practically normal fertility, when the pill is discontinued. This is felt to be due to their relatively low affinity, as compared to the affinity of oestrogen tissue receptors, for which K_a greater than $10^9\ M^{-1}$ were observed. Anyhow, further studies are needed to see if the anti-EE antibodies may have consequences in hormone secretion and in the fertility level.

The role of the anti-EE antibodies in the thrombosis induced by oral contraceptives was established by the correlations observed in the epidemiologic work cited above (Beaumont *et al.*, 1978). But the rarity of thrombosis as compared to the finding of circulating immune complexes remains to be explained. In the present work, the risk of vascular thrombosis is not related to a special variety of anti-EE antibodies. A comparative study on the immune complex composition is now in progress, to find out if they have a different pattern in cases of thrombosis.

Though unexplained, the relation between oral contraceptives, anti-EE antibodies and thrombosis is statistically obvious, and led us to investigate the possible role of other hormones. Antihormone antibodies and thrombosis were found (Table 5) following treatments with didrogesterone, hexestrol and diethylstilbestrol. Of course, the data would have to be confirmed by systematic research, but it must be noticed: (1) that the antibodies are in each case specific of the ingested synthetic hormone; (2) that the risk of thrombosis in patients with prostatic carcinoma treated with diethylstilboestrol is well known. An epidemiological estimation on the frequency of antihormone antibodies in these patients is in progress.

So it is felt that the induction of antibodies to synthetic hormones acting as haptens is a general phenomenon in humans, overlapping the frame of oral contraception by oestro-progestative hormones, and with possible pathological consequences which may not be limited to thrombosis.

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