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HUMAN ALPHA-FETOPROTEIN-FATTY ACID INTERACTION

Claude AUSSEL and René MASSEYEFF

INSERM U2IO,Faculté de Médecine,06034 NICE Cedex,France

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SUMMARY.Human alpha-fetoprotein (AFP) is able to bind arachidonic acid with high affinity $Ka=10^{7}M^{-1}$ and a limited number of binding sites NS=3.Thirty different fatty acids were tested by competition using labelled arachidonic acid as tracer.This experiment demonstrated a great specificity for the fatty acid-AFP interaction.Only polyunsaturated long chain fatty acids were able to bind to AFP with high affinity.The metabolic products of arachidonic acid i.e. prostaglandins presented no affinity for the AFP molecule.

Alpha-fetoprotein (AFP) is a protein found in high concentration in the serum of foetuses and new-borns.It has attracted attention thanks to its reappearence in several pathological conditions in adult,principally during hepatocellular carcinomas(I).Rat and mouse AFPs display high specific binding affinity for estrogens (2,3,4) whereas the homologous protein from other species like that of human do not posses this property (5,6).The biological function of AFP is not known although several experimental observations have suggested various possible roles. The binding of estrogens to AFP indicates that it may be important in the transport of these hormones (7) and it has been suggested that this protein and it has been suggested that this protein protects the embryo against the toxic effect of mother's estrogen on the embryo central nervous system (8).Other workers postulated an immunosupressive role (9,IO) while others have not found this effect (II,I2).More recently we have proposed that AFP play a role in the regulation of ovarian activity (I3,I4).All these roles were mainly based on the estrophilic properties of rat AFP and thus are not transposable to the human system.Recent works by Benassayag et al (I5) and Pineiro et al (I6) have shown that rat AFP binds not only estrogens but also some unsaturated fatty acids (FA).A pioneer work by Parmelee et al (I7) showed that purified human AFP contained some FAs,furthermore Berde et al (I8)using fluorescence spectroscopy have shown that human AFP binds fatty acids with high affinity.This fact made the binding of FAs a common property for AFPs from different species.Recently it was proposed that AFP may play a role

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in the development of the central nervous system (I9,20).This prompted us to study the FA-AFP interaction.

MATERIAL AND METHODS

FATTY ACIDS (FA):They were purchassed from Sigma (St Louis,USA).
-Even carbons straight chain:Capric acid (CI0);Lauric acid (CI2);Myristic acid (CI4);Palmitic acid (CI6);Stearic acid (CI8);Arachidic acid (C20);Behenic acid (C22) and Lignoceric acid (C24).
-Monounsaturated FAs:Palmitoleic acid (CI6:I);Elaidic acid or trans-9-octadecenoic acid (CI8:I);Oleic acid or cis-9-octadecenoic acid (CI8:I); II-eicosaenoic acid (C20:I);Erucic acid or cis-I3-docosenoic acid (C22:I) and Nervonic acid (C24:I).
-Polyunsaturated FAs: Linoleic or cis-cis-9,I2-octadecadienoic (CI8:2); Linolenic or 9,I2,I5-octadecatrienoic acid ((CI8:3);II,I4,I7-eicosatrienoic acid (C20:3);Arachidonic or 5,8,II,I4-eicosatetraenoic acid (C20:4) 5,8,II,I4,I7-eicosapentaenoic acid (C20:5) and 4,7,I0,I3,I6,I9-docosahexaenoic acid (C22:6).

PROSTAGLANDINS:PGE_I ,PGE_2 ,PGF_{Ia} and PGF_{2a} were purchassed from Sigma.

ALPHA-FETOPROTEIN :Pooled human fetal material obtained from legal abortions (I0 weeks pregnancy) was homogenised with a polytron waring blendor at 4°C in 50mM Tris-HCl buffer pH 7.The homogenate were centrifuged for 20 min at 7000 rpm in a Sorvall centrifuge (rotor GS3).Supernatants were pooled and precipitated at 4°C overnight at 40% saturation with ammonium sulfate.After centrifugation,the supernatant was adjusted to 65% saturation with ammonium sulfate and stirred overnight .After centrifugation (30min,9000g) the pellet was dissolved in distilled water and dialysed against 50mM Tris-HCl buffer pH 7.DEAE-Trisacryl M (IBF Pharmindustrie,Villeneuve la Garenne,France) was equilibrated in the same buffer,packed in a 5x24 chromatographic column and washed until stable resistivity of the effluents.The dialysed extract (50ml) was applied to the column and the gel was washed with the equilibration buffer until A_{280nm} returned to the baseline.Elution was then performed with a linear molarity gradient (0 to 0.5M NaCl) total volume I lit, flow rate I00ml/h and monitored for absorbance at 280 nm.Each fraction (6ml) was tested for AFP by immunodiffusion.The fractions with AFP were pooled,concentrated under presure filtration using Amicon Diaflo PMI0 membranes with 25 psi,then submitted to affinity chromatography on anti AFP antibodies coupled to Sepharose 6B (Pharmacia,Uppsala,Sweden).The absorbent was packed in a 2.5x5 cm column and the peaks from DEAE-Trisacryl were applied on this absorbent.The column was then washed with PBS (0.I5M NaCl,pH 7.5).AFP was eluted with 0.I M HCl-Glycine buffer pH 2.8 containing 0.5 M NaCl.After neutralization with I M K_2HPO_4,AFP was dialysed and concentrated.The purity of the protein was verified using electrophoresis in 5% acrylamide gels and immunoelectrophoresis.Goat anti human AFP and goat anti whole human serum immunsera were a gift from G.Pettazzi and B.Ferrua,Laboratoire d'Immunologie,UER de Medecine, Nice.

EQUILIBRIUM DIALYSIS This technique is generally not used for the study of protein-FAs interaction because in I975,Spector (25) said that FAs having a CI2 carbon chain or more does not pass through dialysis membranes.This was not demonstrable in our hands,furthermore,we were able to demonstrate that C20 and C22 fatty acids pass through dialysis membranes and compete with estradiol for the rat AFP binding site (27,28) Taking into account these results we have studied the FA-human AFP interaction using the same technique of equilibrium dialysis.In order to remove most of the endogenous ligands that may be bound to AFP,the

protein (Img/ml) was treated for I5min at 37°C with 5mg of Norit A charcoal (Prolabo,Paris) and centrifuged.One ml of AFP solution (2-I0 ug) was dialysed (dialysis tubing 8/32,Union Carbide Co,Chicago,USA) against I5ml of PBS containing a mixture of radioactive arachidonic acid and unlabelled competitors at 20°C for 24 hrs under continuous rotative agitation.5,6,8,9,II,I2,I4,I5-^{3}H-arachidonic acid (I00Ci/mmol) was purchassed from Amersham,England.The concentration of unlabelled FAs was varied from $I0^{-8}$ to $I0^{-4}$M.Two samples (200ul) from inside and outside the dialysis bag were mixed with Picofluor 30 (Packard,Downers Grove, USA) and radioactivity was counted in a liquid scintillation spectrometer.Association constants were calculated from the concentration of the competitor which reduce by 50% the initial binding of arachidonic acid using the method described by Cheng and Prussoff (2I)

AFP ASSAY : AFP was assayed using the enzyme-immunoassay technique previously published (22) modified as described by Belanger and Masseyeff (23).

RESULTS

Binding of arachidonic acid to AFP. Binding curves were constructed by measuring the amount of bound arachidonic acid at equilibrium in mixtures containing a fixed amount of ^{3}H-arachidonic acid and increasing amount of unlabelled FA.When the data were displayed in a Scatchard plot,an association constant of $IxI0^{7}M^{-I}$ was found (Fig.I).Assuming a molecular weight of 70000 daltons for AFP,the equilibrium dialysis method gives 2.94 to 3.I5 molecules of arachidonic acid bound per molecule of AFP.

Specificity towards FAs

Saturated FAs.The displacement of ^{3}H-arachidonic acid by different saturated FAs is shown in Fig.2.The calculated apparent association

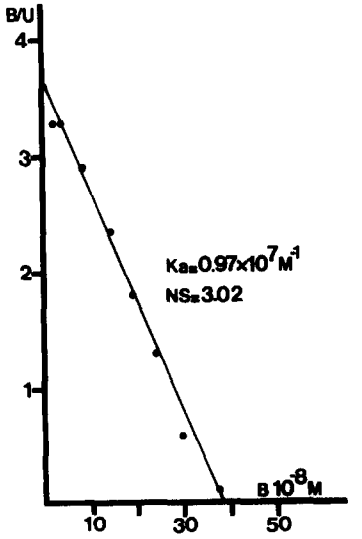

Fig.1 Scatchard plot obtained for the binding of arachidonic acid with human alpha-fetoprotein.

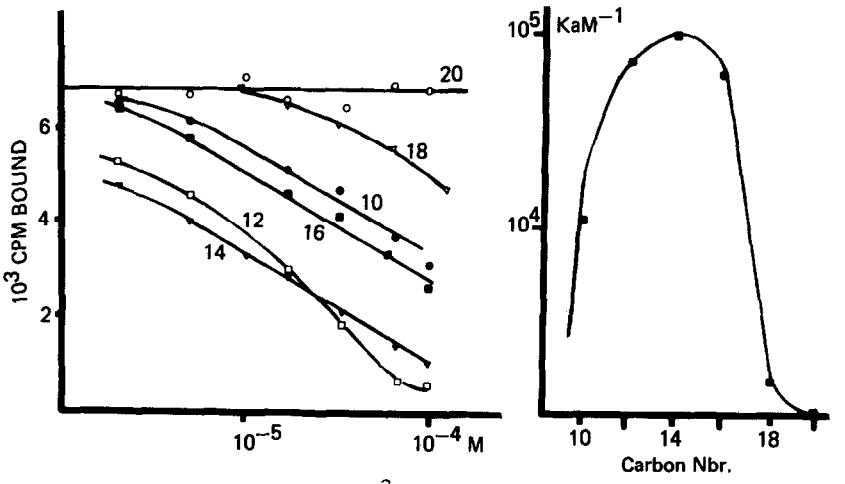

Fig.2 On the left,displacement of ^{3}H-arachidonic acid from AFP by increasing amounts of unlabelled saturated FAs.Capric acid (10) , Lauric acid (12),Myristic acid (14),Palmitic acid (16),Stearic acid (18) ,Arachidic acid (20),Behenic acid (22) and Lignoceric acid (24) On the right,evolution of Ka in function of the number of carbons.

constant varied from $0.4xIO^4M^{-I}$ to $IxIO^5M^{-I}$.FAs having a 20 carbon chain or more did not compete for the FA binding sites.The study of shorter chain FAs showed a maxima for a I4 carbon chain (Fig.2).The Ka values extrapolated from the curves were $0.IxIO^5M^{-I}$,$0.77xIO^5M^{-I}$,$IxIO^5M^{-I}$ and $0.I6xIO^5M^{-I}$ for CIO,CI2,CI4 and CI6 FAs respectively.

Monounsaturated FAs . FAs from CI6 to C24 were used.Fig.3 shows the displacement of ^{3}H-arachidonic acid by these compounds.The Ka values

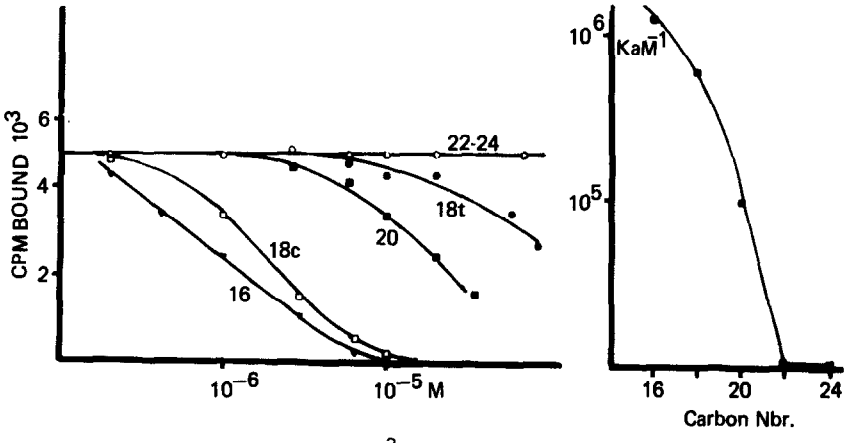

Fig.3 On the left,displacement of ^{3}H-arachidonic acid from AFP by increasing amounts of monounsaturated FAs.Palmitoleic (16),oleic (18) 11-eicosaenoic (20),erucic (22) and nervonic (24) acids.On the right, evolution of the affinity for AFP in function of the number of carbon atoms.

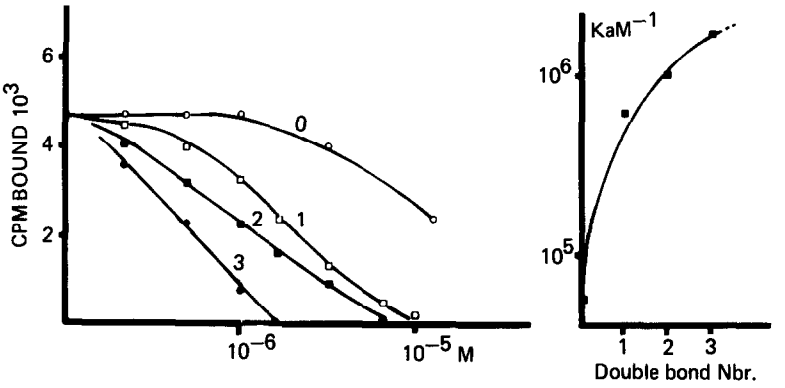

Fig.4 On the left,displacement of ^{3}H-arachidonic acid by increasing amounts of C18 FAs.Stearic (0),oleic (1) ,linoleic (2) and linolenic (3) acids.On the right,evolution of the affinity for AFP in function of the number of double bonds.

obtained from the curves were $IxIO^6M^{-I}$,$0.59xIO^6M^{-I}$,$0.5xIO^5M^{-I}$ for CI6:I, CI8:I and C20:I respectively.FAs having more than 20 carbons did not compete with arachidonic acid.Plot of Ka versus the number of carbon atom in the chain showed an inverse relationship.

Cis-Trans isomers.The apparent association constant was $0.59xIO^6M^{-I}$for the cis isomer of CI6:I while it decreased to about $IxIO^4M^{-I}$ for the trans isomer (Fig.3).

Effect of the number of double bonds

CI8 series.A striking influence of the number of double bonds in the CI8 series is shown in Fig.4.CI8:0 bind to AFP with a Ka of about $6xIO^4M^{-I}$ and the introduction of I to 3 double bonds developped the capacity of binding to the AFP molecule.The apparent association constants calculated were $0.59xIO^6M^{-I}$,$IxIO^6M^{-I}$ and $0.2xIO^7M^{-I}$ for CI8:I, CI8:2 and CI8:3 respectively.

C20 series.A similar conclusion could be derived from the study of C20 FAs.In this series FAs having 0 to 5 double bonds were available.The Ka values were $IxIO^5M^{-I}$,$I.6xIO^6M^{-I}$,$4xIO^6M^{-I}$,$IxIO^7M^{-I}$ and $0.66xIO^7M^{-I}$ for C20:0,C20:I,C20:2,C20:3,C20:4 and C20:5 respectively.Thus there was no further effect of the number of double bonds when their number was supperior to 4 (Fig.5).

C22:6 .In the C22 series,only the C22:6 was available.This FA was shown able to bind to AFP with an association constant $Ka=2xIO^7M^{-I}$ (Fig.6).

Prostaglandins .PGE_I,PGE_2 ,PGF_{Ia} and PGF_{2a} were tested (Fig.6).None of these compounds were able to compete with arachidonic acid even at the highest concentration tested ($IO^{-5}M$).

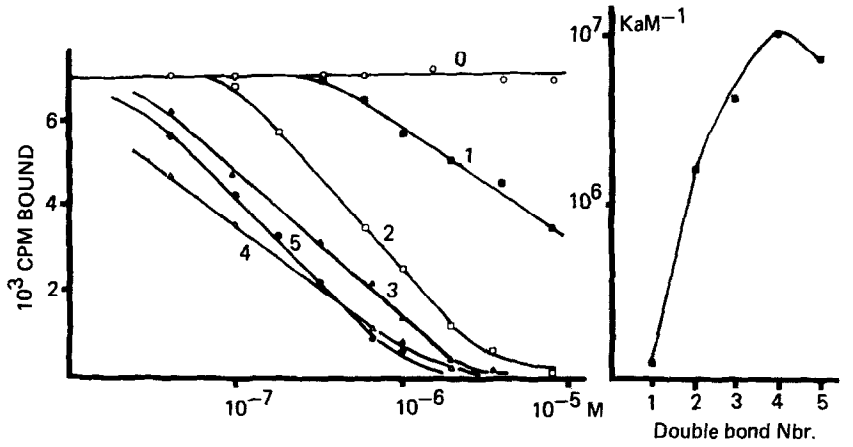

Fig.5 On the left,displacement of ^{3}H-arachidonic acid from AFP by C20 FAs. Arachidic (0),11-eicosaenoic (1),11,14-eicosadienoic (2),11,14,17-eicosatrienoic (3),arachidonic (4) and eicosapentaenoic (5) acids. On the right,evolution of the affinity in function of the number of double bonds.

DISCUSSION

The binding of FAs by rat AFP was first demonstrated by Savu et al (I5).These authors described the general trends that we have reported here,i.e.polyunsaturated FAs had the highest Ka. A possible interaction of FAs with human AFP was suspected by Parmelee et al (I7) that showed the presence of FAs in purified AFP.In the present work we have tested the binding properties of human AFP and showed i) AFP bind preferentially long chain polyunsaturated FAs.The affinity constant increased with the number of double bonds in the CI8 and C20 series and probably in the C22 series.C22:6 was found to be the best ligant for AFP among the 30 FAs tested. ii)The number of binding sites on AFP was found to

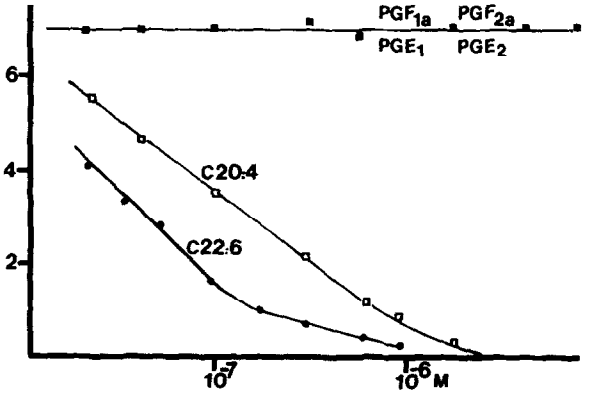

Fig.6 Displacement curves obtained with arachidonic acid (C20:4) and docosahexaenoic acid (C22:6) .Prostaglandins presented no particular affinity for AFP.

be equal to 3.The 3 binding sites presented the same affinity as judged by the straight line of the Scatchard plots.This number of binding sites confirm the results obtained by Parmelee et al (I7) that showed that AFP purified in mild conditions contained 2 to 3 mol FAs/mol AFP. Furthermore,Berde et al (I8) using fluorescence spectroscopy demonstrated the presence of 3 binding sites on the molecule.Gorin et al (24) found that the genes of both mouse AFP and albumin derived from a common ancestral gene.This ancestral gene being triplicated during the evolution process.The binding of FAs to both albumin and AFP can be considered as un argument to support this theory.The successive mutations having transformed the ancestral gene in order to give two different proteins having similar binding properties but different specificity as albumin bind saturated FAs while AFP prefer the unsaturated ones.More over the presence of three binding sites on AFP is in good agreement with the theory of the triplication of the ancestral gene.This fact was not maintained for the albumin that present 8-9 FAs binding sites (25,26).

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