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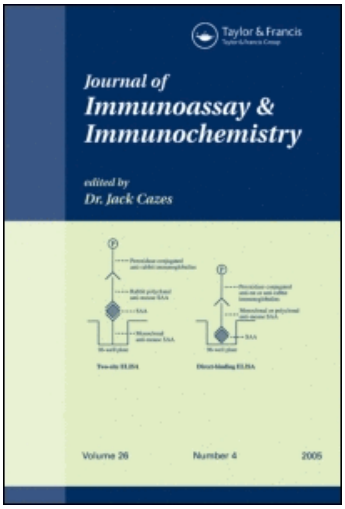

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Production of a New Monoclonal Antibody Specific to Human Des-Gamma-Carboxyprothrombin in the Presence of Calcium Ions. Application to the Development of a Sensitive ELISA-Test

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JOURNAL OF IMMUNOASSAY, 16(2), 213-229 (1995)

PRODUCTION OF A NEW MONOCLONAL ANTIBODY SPECIFIC TO HUMAN DES-GAMMA-CARBOXYPROTHROMBIN IN THE PRESENCE OF CALCIUM IONS. APPLICATION TO THE DEVELOPMENT OF A SENSITIVE ELISA-TEST.

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ABSTRACT

In order to explore pathologies possibly associated with vitamin K deficiency, several monoclonal antibodies (mAbs) were produced against human Desgamma-Carboxy-Prothrombin (DCP). One of these mAbs, designated C4B6, detected DCP forms in the presence of Calcium ions, confirmed by comparison with the patterns of two electrophoretic techniques : Affino-Immuno-Electrophoresis (CAIE) and Polyacrylamide Gel Electrophoresis followed by Electro-blotting (PAGE-Blot).

An Enzyme-Linked-Imunosorbent Assay (ELISA) using mAb C4B6 has been developed, optimized and standardized. It has proven to be specific for DCP forms and has a minimum sensivity of 0.156 A.U/ml.

(KEY WORDS : Monoclonal antibody, Des-gamma-carboxyprothrombin, PIVKA II, ELISA, Vitamin K, Calcium).

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INTRODUCTION

Prothrombin or coagulation factor II is a glycoprotein binding to bivalent metal ions such as Ca^{2+}. Its post-translational activation (gamma-carboxylation) is vitamin K-dependent. Many other proteins have analogous properties. These are, among others, coagulation factors VII, IX, X, hemostasis regulation proteins C, S, M, Z and proteins occurring in calcified tissues such as osteocalcin or Bone Gla Protein (BGP), Matrix Gla Protein (MGP) and Plaque Gla Protein (PGP). After their maturation, all contain gamma-carboxyglutamic acid residues (Gla) localized in a segment of polypeptide near the amino-terminus termed the "Gla domain". The conversion of some glutamic acid residues to Gla is catalysed by vitamin K-dependent carboxylase. Vitamin K functions as an obligatory cofactor during the enzymatic reaction. This carboxylation induces the Ca^{2+}-binding capacity of "Gla-proteins" and that confers, in particular, the ability to bind acidic phospholipid vesicles, a requisite step of coagulation (1).

In the absence of vitamin K or in the presence of vitamin K antagonists, and more generally, during any modification of the carboxylation cycle, abnormal non functional forms (more or less decarboxylated) of these proteins circulate in the blood and can become markers of such modifications. Des-gamma-Carboxy-Prothrombins (DCP or PIVKA II) are the non-functional forms of Prothrombin.

Different techniques of prothrombin detection and quantification have been developed during the last few years, of which the most attractive is the method using mAbs. Initially, we developed an original electrophoretic technique derived from CAIE, which we named PAGE-blot (2) and used later as a reference technique. We subsequently produced several anti-prothrombin-mAbs, one of which has been shown to be specific for at least one DCP form in the presence of Ca^{2+} ions. We have developed an ELISA test using this antibody and examined its specificity and affinity by comparing it with electrophoretic techniques (CAIE and PAGE-blot). This study was carried out on approximately 200 plasma samples both from healthy donors and patients with possible vitamin K deficiency.

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MATERIALS AND METHODS

1. Electrophoresis

Two techniques were chosen as references.

- CAIE was the classical electrophoretic technique (3). This crossed electrophoresis in agarose gel was performed in the presence of calcium lactate (4). Sensitivity was about 1 µg/ml.

- PAGE-blot derived from CAIE was developed in the laboratory. It consisted of electrophoresis performed in polyacrylamide gel in the presence of calcium lactate (2 mM) followed by immunoblotting onto nitrocellulose and visualization realised with anti-normal prothrombin serum (2). If DCP was present in plasma samples, two bands were observed on the blot. This method was correlated to CAIE and found to be ten times more sensitive than CAIE (5).

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2. Plasma samples

Blood collections were made in different hospital laboratories under medical supervision either in out-patients department or during hospitalization. Blood was collected onto citrated silicone tubes. After centrifugation, plasma was stored at -70°C until utilization.

Blood sampling consisted of :

- Healthy donors used as the negative control population
- Patients receiving oral anticoagulant treatments representing positive control population (pool used to construct a standard curve)
- Cystic fibrosis (CF) children (n=89)
- Patients with hepatocarcinoma (HCC) or/and cirrhosis (C) (n=71)
- Full term breast-fed infants who were given 2 mg of vitamin K1 orally the first day of their life (n=37)
- Premature breast-fed infants who were given 2 mg of vitamin K1 orally every week during the first month (n=21).

3. Prothrombins

Normal prothrombin was from Diagnostica Stago (Asnières, France) or Sigma (Saint Quentin Fallavier, France).

Fractions with a high DCP content (>80%) used for screening of supernatants were obtained from a anticoagulant plasma pool, centrifuged for 10 min at 2500 rpm , filtered on 0.2 mm and adsorbed onto $BaCO_3$ (2). Benzamidine hydrochloride 1 mM (Sigma) and Sodium azide 0.02 % (Sigma) were then added. The purification involved two successive chromatographic steps : ion exchange chromatography on DEAE-Sephacel (Pharmacia, Uppsala, Sweden) using pH 7.6 buffer (20 mM Tris, 200 mM NaCl) followed by gel filtration chromatography using AcA-44 Ultrogel (Pharmacia) with a pH 7.6 elution buffer (20 mM Tris, 500 mM NaCl). Collected fractions were tested, pooled and concentrated using ultrafiltration with CX-3C immersible membranes (Millipore, Saint Quentin Yvelines, France), and finally stored at 4°C.

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4. Monoclonal antibodies

To obtain fractions rich in DCP, the first purification chromatographic step onto DEAE Sephacel was performed as described above. The collected fractions were tested using direct-ELISA (see below) and the positive fractions were pooled and concentrated. An electrophoresis on a 10 % polyacrylamide gel using a 10 mM calcium lactate buffer was performed. Gel zones containing DCP identified by colouration of a control strip were cut and ultrasonicated into PBS (0.1 M phosphate buffer saline, pH 7.4) for 20 sec.

Balb/c mice were then immunized with this preparation mixed with Freund's complete adjuvant (Sigma) for the first injection. For subsequent injections Freund's incomplete adjuvant (Sigma) was used in the same proportion. Three days after administering a booster injection, splenocytes of the immunized mice were taken and fused with mouse non-secreting myeloma cells (line SP2O) according to standard procedures (6).

Growing hybridomas were screened by ELISA methodology and positive supernatants were cloned by limiting dilution. Nunc Immunoplates-

Maxisorp (Roskilde, Denmark) were coated with 100 µl of fraction high in DCP (1 µg/ml PBS). Cultured supernatants were incubated in the antigen coated wells at 37°C for 30 min. After PBST (phosphate buffer saline containing 0.1% tween 20) washing, horseradish peroxidase labelled goat anti-mouse immuno-globulin (Ig G + Ig M) (Jackson Immuno-Research, Baltimore, PA) was added diluted 1:5000 in PBST and the plates incubated at 37°C for 20 min. Enzyme activity was measured using 100 µl of 0.045 % H_2O_2 in 0.1 M Citrate buffer pH 5.0 containing 3 mg/ml of O-phenylenediamine (OPD). The reaction was stopped 10 min later by addition of 0.5 N H_2SO_4 (100 µl/well). Optical density (OD) was measured at 492 nm using a Behring ELISA Processor II reader (Hoechst Behring, Rueil-Malmaison , France).

In addition, a second direct-ELISA methodology was developed using normal prothrombin or commercial albumin for plate coating as albumin is the principal contaminant during the purification step.

Finally, from the 18 clones preselected, 5 antibodies were purified from ascites fluid using ion exchange chromatography on gel (Mem Sep 1010-DEAE, Millipore) balanced in Tris 20 mM buffer pH 8.0. Elution was carried out using an NaCl linear gradient between 0 and 1 M.

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5. Selection of the C4B6 antibody

In order to determine the specificity of the mAbs obtained, different PAGE-blots were performed under denaturing or native conditions. Plasma samples from healthy donors or patients and normal or abnormal prothrombin were used.

A series of cross-reactivity assays were carried out by direct-ELISA using potential cross-reacting agents such as factors VII (Sigma), IX (Stago), X (Sigma), thrombin (Sigma) or protein C (Stago) coated to the plate (0.2 µg protein / ml).

To determine more exactly the clones' affinity for native plasma DCP, an indirect-ELISA methodology was developed. 10 µg/ml of rabbit Ig G anti-human normal prothrombin purified from commercial antiserum (Diagnostica Stago) was adsorbed onto the plate wells after dilution in 50 mM carbonate-

bicarbonate buffer pH 9.6. Following plate washing with PBST, negative and positive control plasma pools adsorbed or non adsorbed onto $BaCO_3$ were diluted 1:100 in PBST and added to wells for 60 min at 37°C. Pure supernatants or ascites fluids were diluted and incubated at 37°C for 60 min and horseradish peroxidase labelled rabbit anti-mouse (Ig (G+M)) (Jackson Immuno-Research), diluted 1:5000 into PBST was then added for 30 min at 37°C. OPD was used as revelation system.

A similar ELISA methodology has been developed using TBST (20 mM Tris, 150mM NaCl, 0.1% Tween 20, pH 7.4). The antibody tested was previously diluted in 10 mM EDTA (TBST-EDTA) or 10 mM calcium lactate (TBST-Ca) buffers.

6. Development of ELISA for DCP (C4B6-ELISA)

An indirect ELISA has been developed using purified C4B6 mAb coated on plate wells. In this case, plasma samples were previously diluted in TBST-Ca (10 mM). The anti-normal prothrombin serum (Diagnostica Stago) was added before the goat anti-rabbit conjugate (Jackson Immuno-Research). Detection was realised with OPD. Optimisation of different parameters (choice of solutions' concentrations, incubation times...) and controls (outlined in "Results" section) allowed realisation of the specific anti-DCP ELISA test.

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7. Controls and standards

The negative control was represented by a pool of 8 plasma samples chosen from the population of healthy donors. Positive control (standard) consisted of a pool of 20 plasma samples from patients given oral anticoagulant therapy. In order to develop a standard curve, positive control was successively diluted 1 in 2 in negative control to obtain the same OD as negative control. These different standard solutions were then rediluted 1:100 in TBST-Ca.

To standardize the test, DCP concentration in positive control pool (standard) was determined both by Rocket Immuno-Electrophoresis (7) and indirect-ELISA techniques before and after adsorption onto $BaCO_3$ (2) and compared with solutions of commercial prothrombin. Commercial prothrombin was previously quantitated by BCA-1 kit (Sigma) using an indirect-ELISA

similar to the methodology exposed in part 5: sheep immunoglobulin fraction anti-human prothrombin (Serotec, Oxford, England) and horseradish peroxidase labelled rabbit anti-sheep (Jackson Immuno-Research) were used.The DCP concentration of standard was 40 ± 2 µg/ml depending on techniques we used.

8. Asserachrom-PIVKA II kit:

A commercial ELISA (Asserachrom-PIVKA II, Diagnostica Stago) with another monoclonal antibody specific to DCP (named P1-2B9) was used, on the one hand to dose DCP in positive control and, on the other hand to compare affinity and specificity of P1-2B9 and C4B6 for native DCP forms.

To know whether C4B6 and P1-2B9 antibodies recognized the same DCP epitope, biotinylated C4B6 was substituted for usual conjugate in Asserachrom-PIVKA II kit, and our buffers (with or without calcium ions) were substituted for usual buffers.

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RESULTS

1. Monoclonal antibodies

Screening of hybridomas was carried out using direct-ELISA with alternatively the fraction high in DCP or the commercial normal prothrombin as coating. Of the 18 mAbs preselected, 10 reacted with these antigens. Eight other mAbs recognized only the fraction high in DCP.

Firstly, we studied these 8 mAbs, hoping that they would be specific of native DCP forms. They were tested by different PAGE-blots and indirect-ELISAs using comparatively plasma samples' pools with and without DCP. No interesting results were obtained. Although these mAbs did not recognize albumin in direct-ELISA (albumin was the main contaminant detected during purification controls of the fraction high in DCP we used), we assumed that they reacted with other contaminants of the DCP fraction or with modified epitopes of DCP (epitopes modified during the electrophoretic step of purification performed before immunization).

TABLE 1

REACTIVITY DIFFERENCES OF THE 10 CLONES*
using indirect-ELISA in the presence of calcium lactate or EDTA (10 mM)

	Indirect-ELISA			
	EDTA		Ca^{2+}	
clones	**P****	**DCP*****	**P****	**DCP*****
A5A5	(+)	(+)	-	(+)
C8G9	+	(+)	+	+
B4B1	++	+	++	+
C1A7	-	-	-	-
C4B6	**+++**	**+++**	**-**	**+++**
C7C2	+++	+++	+	+++
C4G7	++	++	+	++
C3F3	++	+	++	+
C4B10	-	-	-	-
C5G9	++	++	(+)	++

Key : * clones that reacted with both normal and abnormal prothrombin using direct-ELISA and PAGE-blot. **P****, prothrombin of negative control (healthy donors plasma samples). **DCP*****, abnormal prothrombin of positive control (plasma samples of patients receiving oral anticoagulant treatments, preadsorbed on $BaCO_3$).

$OD\Delta > 1.5$, +++ ; $1 < OD\Delta < 1.5$, ++ ; $0.5 < OD\Delta < 1$, + ; $OD\Delta < 0.2$, -.

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We consequently investigated the 10 other mAbs. The different PAGE-blots did not give complementary data with regard to a particular antibody selection : each mAb revealed both normal prothrombin and DCP (with variable intensity) on blot. On the other hand, the indirect-ELISAs developed allowed us to examine their affinity towards native prothrombins and to confirm their specificity prestudied by direct-ELISA (Table 1). In the presence of calcium, a discriminating answer of one clone between functional and non functional

prothrombins was observed. Clone C4B6 appeared to be interesting as it was specific for DCP forms only in the presence of calcium ; it recognized normal and abnormal prothrombin in the presence of EDTA. Of the supernatants tested for cross-reactivity, only C8G9 and C7C2 recognized factor X in addition to prothrombin using direct-ELISA. None of the antibodies recognized thrombin or albumin.

2. C4B6-ELISA : Optimisation and standardization

Each ELISA parameter was tested in order to obtain the smallest possible OD with the negative control and the optimal OD difference (ODΔ) between the positive and negative controls.

In order to standardize the assay, positive control pool (standard) was quantitated by Rocket Immuno-Electrophoresis and ELISA assays as explained previously. A value of 40 ± 2 µg/ml of DCP was established. But using Asserachrom-PIVKA II, DCP concentration of the same standard was 4800 ng/ml. This is the reason why we preferred to express our results in arbitrary unit (A.U). So the values obtained for the successive standard dilutions ranged between 40 and 0.156 A.U/ml (limit of detection). According to the range of negative control pool (healthy adults plasma samples), the cut off value was put to 0.312 A.U/ml.

Based on these results, an ELISA mode (named n°1) was developed. Corresponding experimental mode is summarized in table 2. ELISA n°1 was able to assay samples containing between 0.312 and 5 A.U of DCP /ml. Applying the test to plasma samples high in DCP allowed us to shorten the total assay time by changing incubation times and concentrations of the reagents (ELISA n°2, exposed in table 2).

3. Reproductibility

Figure 1 shows the two typical curves for standard solutions of DCP using the ELISA protocols described in table 2 in the range 40 A.U/ml to 0.312 A.U/ml. The coefficients of variation (CV) for three reference standard dilutions

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TABLE 2

METHODS OF THE TWO ANTI-DCP ELISA TESTS

	ELISA n°1	**ELISA n°2**
Coating : C4B6 mAb	10 µg/ml 1 night, 37°C	10 µg/ml 1 night, 37°C
Antigen : plasmas	/100 in TBST-Ca 60 min, 37°C	/100 in TBST-Ca 30 min, 37°C
Anti-serum : AS	/10000 30 min, 37°C	/10000 15 min, 37°C
Conjugate : Anti-AS	/5000 15 min, 37°C	/10000 15 min, 37°C
Revelation : OPD	3 mg/ml 10 min	3 mg/ml 10 min
Stop : H_2SO_4	100 µl/well	100 µl/well

Key : AS, rabbit anti-serum against human normal prothrombin; Anti-AS, goat conjugate anti-rabbit.

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(within run precision of the assay) were less than 10 % (Table 3). The between run precision of the assay was also tested six times using 57 plasma samples of patients with hepatocarcinoma and/or cirrhosis. In this case, the CV values were also less than 10 % ; three values are given as example in table 3.

4. Specificity of the ELISA-system

The tests developed for the selection of the clones allowed us to examine the specificity of antibody C4B6 towards prothrombin and its lack of affinity for other Gla-proteins. Compared to a value of 100 % for cross-

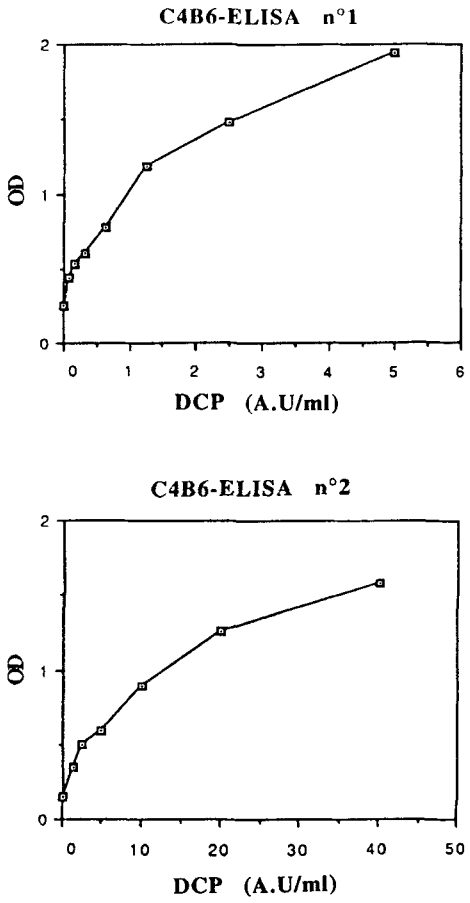

FIGURE 1: ELISA-C4B6 n°1 and n°2 STANDARD CURVES: Optical Density (OD units) in function of DCP (A.U/ml)

reactivity with prothrombin, the other Gla-proteins tested presented values less than 5 %.

In addition previous CAIE and PAGE-blot results were compared to ELISA using 161 plasma samples from patients with possible vitamin K deficiency (21 premature infants, 37 full-term infants, 89 CF children and 14 patients with HCC). Only 4 patients with HCC presented DCP using CAIE. All

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TABLE 3

WITHIN-ASSAY and BETWEEN-ASSAY VARIATIONS

Within-assay (n=8)			**Between-assay** (n=6)		
Mean (A.U/ml)	SD (A.U/ml)	CV (%)	Mean (A.U/ml)	SD (A.U/ml)	CV (%)
8.442	0.737	8.7	3.908	0.154	3.9
4.200	0.212	5.1	1.642	0.133	8.2
2.067	0.157	7.6	0.958	0.053	5.6

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15 DCP positive samples using PAGE-blot (2/37, 2/89, 11/14) resulted in a positive ELISA result (>0.312 A.U/ml). Out of 146 samples which were negative using PAGE-blot, 20 were positive using the ELISA test (20/89 CF children).

Diagnostica Stago have developed an ELISA against DCP using mAb P1-2B9. In collaboration with Pr. Guillin's team, we compared the Diagnostica Stago kit and our C4B6-ELISA in terms of specificity and affinity using 57 plasma samples from patients with hepatocarcinoma and/or cirrhosis. A good correlation was found between the two methods. The correlation coefficient (r) was 0.884 ($p<0.0001$). Figure 2 illustrates the regression line concerning low values (n=52).

Modifying Asserachrom-PIVKA II protocol allowed us to compare C4B6 and P1-2B9 antibodies. P1-2B9 was specific of DCP forms in the presence or in the absence of calcium. Furthermore, biotinylated C4B6 could substitute for usual conjugate, which proved that C4B6 mAb did not recognize the same epitope than P1-2B9 but could recognize the same DCP forms.

DISCUSSION

Nowadays, several techniques for detection and measurement of DCP in blood may be used. Indirect methods, such as Rockets Immuno-

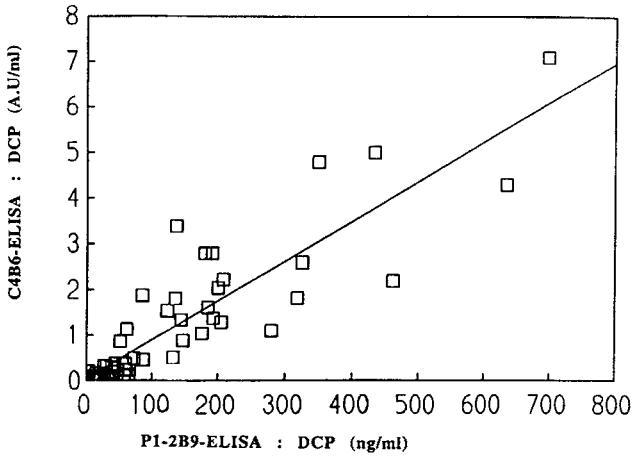

FIGURE 2: CORRELATION between P1-2B9-ELISA (Diagnostica Stago) and C4B6-ELISA.
Illustration of regression line obtained with 52 low values (DCP<800 ng/ml) of plasma samples from patients with hepatocarcinoma or/and cirrhosis.
slope = 0.8686 ± 0.0648
y intercept = 2.2566 (when x=0)
x intercept = -2.5981 (when y=0)
r = 0.8844.
The SD of residuals from the line (Sy.x) = 71.8511
P<0.0001.

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Electrophoresis (RIE) (7) and activation using staphylocoagulase (8) are reliable and quite sensitive, but they require a step of pre-adsorption on salts or on gels and bivalent ions. They may also present partial adsorption of DCP. Of the direct methods, CAIE (4) has until now been used because of its reliability and its simplicity (9), even though it is a qualitative technique, with limited sensitivity (1 µg/ml). As a second reference method we developed in our laboratory a PAGE-blot methodology (2) which correlated well with the CAIE and demonstrated greater sensitivity (100 ng/ml) (5).

Over the last two decades, monoclonal antibodies have been developed and used. Furie et al. 1978 (10) isolated for the first time sub-populations of polyclonal antibodies from rabbit sera by affinity chromatography. Using RIA,

they then developed a sensitive methodology of detection for these DCP forms. However, obtaining these sub-populations of antibodies was very difficult. Motohara et al, 1985, (11) presented an ELISA test using the MU3 mAb. Its sensitivity was limited to 0.13 AU (1 AU = 1 µg/ml of purified material). The methodology was optimized by Eisaï Co (Tokyo, Japan). Tsai et al, 1990, (12) published results comparing staphylocoagulase methodology and MU3-ELISA. Church et al., 1989, (13) succeeded in developing an ELISA test using the H11 mAb directed against protein C but it also recognized factors II, VII and X. Finally, Amiral et al, 1991, (14) developed an ELISA test (Asserachrom PIVKA II) using the P1-2B9 mAb (Stago) which had a limit of sensitivity of about 0.2 ng/ml.

Among the monoclonal antibodies against human prothrombins produced by us, only one (C4B6) was specific of des-gamma-carboxy-prothrombins because of its total difference in reactivity in the presence or absence of Ca^{2+} ions using the indirect-ELISA methodology. Whereas in the absence of Ca^{2+} ions both normal and abnormal prothrombins were recognized, in the presence of Ca^{2+} only one or more of the DCP forms were detected. We therefore developed, optimized and standardized an ELISA test using this mAb to reliably and sensitively detect DCP forms in plasma. CAIE, PAGE-blot and ELISA-C4B6 were found to agree in the limiting range of the electrophoretic techniques. Indeed, no PAGE-blot positive samples were missed by ELISA-C4B6. No cross-reactivity with other Gla-proteins was observed. The ELISA was sensitive, fast (less than 2 hours) and requires a small serum volume (5 µl), an important factor in the neonatology field.

Studies performed by Church et al (1989) proved that recognition of the epitope by the H11 mAb does not directly depend on the presence of Gla residues. It depends mostly on a phenomenon induced by Ca^{2+} ions on the normal prothrombin and not induced on the abnormal forms. According to Church et al. (13) this phenomenon consists of a conformational transition which inhibits the linkage between the antibody and the Gla region of the normal prothrombin. The mechanism of recognition of our C4B6 is similar. Both are specific conformation antibodies. However the epitope is different, since the respective specificity towards Gla-proteins are different.

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With regard to P1-2B9 (Stago) antibody, its specificity does not depend on the presence of Ca^{2+} ions. Our results showed that P1-2B9 and C4B6 did not recognize the same epitope, but also that C4B6 could react with DCP forms recognized by P1-2B9. In addition, results using comparatively C4B6 and P1-2B9 were well-correlated (r=0.884).

With regard to a newborn population (n=57 cord blood samples), Eisaï (MU3), Stago (P1-2B9) and C4B6 tests detected the same DCP positive samples with a few exceptions (15). However slight differences might be observed in the intensity of the response. In consequence two important questions may be asked: do these antibodies detect the same forms of DCP ? might the differences be related to particular pathologies ? DCP presents indeed different forms dependent on the degrees of carboxylation (15). The ELISAs we used detect probably only a fraction of the total DCP forms present in the plasma samples. Tsai et al (12) reported the underevaluation (about only 1%) of the true total DCP concentration using MU3 antibody. Moreover, Amiral et al (15) proved that P1-2B9 reacted preferentially with 3-5 Gla DCP forms. In this study, we noted that 1AU of DCP detected in our standard was equivalent to 1µg measured with electrophoretic or ELISA techniques using polyclonal antibodies, and only equivalent to 120 ng DCP measured with Stago kit. This is the reason why we preferred to express our results in AU.

From a clinical point of view, use of a specific mAb and ELISA methodology may result in increasing the sensitivity and the reliability of the dosages. However, is enhancement of the sensitivity required as very low levels of circulating DCP forms are not necessarely significant and may not be correlated with the onset of vitamin K deficiency ? Fixing appropriate cut-off values, maybe dependent on the nature of pathologies, seems to be an important question.

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