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► **To cite this version:**

Mimi Høier-Madsen, Jan Holm, Steen Ingemann Hansen. Alpha-isoforms of soluble and membrane-linked folate binding protein in human blood. *Bioscience Reports*, 2008, 28 (3), pp.153-160. 10.1042/BSR20070033 . hal-00479294

**HAL Id: hal-00479294**

**<https://hal.science/hal-00479294>**

Submitted on 30 Apr 2010

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# ALPHA – ISOFORMS OF SOLUBLE AND MEMBRANE-LINKED FOLATE BINDING PROTEIN IN HUMAN BLOOD

by

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**Running title:** Folate Binding Proteins in Human Blood

## ABSTRACT

The high affinity folate binding protein/ folate receptor (FBP/FR) is expressed in three isoforms. FR alpha and beta are attached to cell membranes by hydrophobic glycosylphosphatidyl inositol (GPI) anchors, while FBP gamma is a secretory protein. Mature neutrophil granulocytes contain a non-functional FR beta on the surface, and in addition nanomolar concentrations of a secretory functional FBP (29 kDa) can be present in the secondary granules. A statistically significant correlation between the concentrations of functional FBP, probably a gamma isoform, in granulocytes and serum supported the hypothesis that serum FBP (29 kDa) mainly originates from neutrophils. The presence of FBP/FR alpha isoforms were for the first time established in human blood using antibodies specifically directed against human milk FBP alpha. The alpha isoforms identified on erythrocyte membranes, and in granulocytes and serum only constituted an almost undetectable fraction of the functional FBP. The alpha FBP in neutrophil granulocytes was identified as a cytoplasmic component by indirect immunofluorescence. Gel filtration of serum revealed a peak of alpha FBP (> 120 kDa) which could represent receptor fragments from decomposed erythrocytes and granulocytes. The soluble FBPs may exert bacteriostatic effects and protect folates in plasma from biological degradation, while FRs on the surface of blood cells could be involved in intracellular folate uptake or serve as signal proteins. The latter receptors have also been used for therapeutic targeting in malignancy.

### Key Words:

Folate binding proteins / Folate receptors in human blood

Alpha isoforms in cells and serum

Neutrophil granulocytes

Erythrocytes

Serum

## INTRODUCTION

A high-affinity folate binding protein (FBP) was first identified in cow's milk (1-3). The protein has been purified and sequenced; it consists of 222 amino acids with a molecular weight of 30 kDa based on amino acid composition and carbohydrate content (4). FBP purified from human milk has a similar molecular weight as estimated from its amino acid composition, carbohydrate content and sequence homology with FBP from cow's milk (5). The purified human milk FBP exists in two forms: one is water-soluble and elutes at 27 kDa on gel filtration and one is equipped with a hydrophobic glycosyl phosphatidyl inositol (GPI) residue and forms micellar aggregates with Triton X-100 eluting at 100 kDa (5,6). The micellar FBP is transformed to the water-soluble FBP after enzymatic cleavage of the GPI tail by phosphatidylinositol-specific phospholipase C, PI-PLC (6).

The ubiquitous existence of FBPs in mammalian cells, tissues and body fluids is now well-established (1,2). Three isoforms of FBP, alpha, beta and gamma, are expressed on chromosome 11q13 in humans (7,8). Only the former two isoforms can be equipped with a GPI tail (9), which enables them to be anchored to plasma membranes as membrane-bound folate receptors (FRs) susceptible to enzymatic cleavage by PI-PLC or proteases. FR alpha is normally expressed in secretory epithelial cells and overexpressed in certain epithelial tumor cells, e.g. ovarian carcinoma (10); its amino acid sequence is homologous to that of human milk FBP which represents the soluble form of FR alpha (5,11).

The presence of soluble and membrane-linked FBPs in human blood cells and plasma have been reported. The outer membrane surface of erythrocytes (12,13) and neutrophil granulocytes contain FRs (14-16); in addition the secondary cytoplasmic granules of neutrophils contain a high-affinity FBP (17-20). The membrane receptor of erythrocytes immunoreacts with polyclonal antibodies against human placenta FR (12), which is a mixture of alpha and beta isoforms (21), while that of neutrophil granulocytes is a beta isoform (22). The water-soluble FBP in neutrophil granules is supposed to be a gamma isoform without GPI residue and most likely a source of origin of plasma/serum FBP (23,24).

As yet, no alpha isoforms of FBP/FR have been identified in human blood. A major issue of this report has therefore been to establish whether these isoforms are present in human blood. For this purpose we reviewed earlier data on FR/FBP in human blood, and employed a combination of radioligand (tritiated folate) binding and immunotechniques involving a polyclonal antibody against purified alpha FBP from human milk (25). The latter antibody exhibits an immunoreactive profile similar to that of the monoclonal antibody, MOv18. Like MOv18 it only immunoreacts, as evidenced by immuno precipitation, immunoblotting, ELISA and immunohistochemistry, with FR alpha, e.g. in serous

ovarian adenocarcinoma (7,10,11,26 ,27) ; normal ovarian tissue where FR beta prevails (7) shows no immunoreactive response (27).

## MATERIALS and METHODS

**Proteins and antibodies:** FBPs were purified from human and bovine milk as previously described (4,5). Polyclonal antibodies against human and bovine milk FBPs were raised in rabbits (25,28). Rabbit antibodies against human lactoferrin (A 0186) and FITC labelled Ig fraction of swine anti-rabbit serum (F 0205) were from DAKO. Rabbit serum was from State Serum Institute.

**Radiochemicals:** [<sup>3</sup>H] Folate with a specific activity of 26-45 kCi/mol was supplied by Amersham International Ltd. Amersham, U.K.

**Preparation of hemolysate from erythrocytes:** Hemolysates were prepared as previously reported in a study dealing with binding of tritiated folate to hemoglobin (29). Briefly, specimens of EDTA stabilized venous blood were drawn from healthy volunteers with normal hematological parameters (hemoglobin, RBC count, MCV, WBC count, and differential count). The plasma and buffy coat layer was discarded after gentle centrifugation (1500 x g) for 15 min, and erythrocytes were washed several times in a volume of isotonic NaCl equal to the plasma volume to remove contaminating plasma. The washed erythrocytes were finally diluted with a volume of distilled water equal to the plasma volume and subjected to repeated freeze-thaw procedures. Aliquots of hemolysates were dialysed overnight against 0.17 M Tris-HCl buffer of pH 7.4 before analysis to remove endogenous folate.

**Preparation of leukocyte lysates:** Specimens of EDTA stabilized venous blood were drawn from healthy volunteers with normal haematological parameters (cf above), leukocytes harvested, washed several times in isotonic NaCl to remove contaminating plasma and then subjected to repeated freeze-thaw procedures (19,20). The cells were homogenized and solubilized in 1 g/l Triton X-100, dialysed against 0.17 M Tris HCl of pH 7.4 for 24 h to remove endogenous folate, and finally centrifuged at 2000 x g for 15 min (19,20). Aliquots of the supernatants were used for analysis.

**Preparation of pooled sera:** Pooled sera were prepared as previously reported (30). Briefly, sera were pooled, dialysed overnight against 0.05 M imidazole buffer of pH 6.3 /30 mM NaCl to remove endogenous folate and then centrifuged at 1000 x g for 15 min. The supernatant was used for analysis.

**Radioligand binding:** Aliquots of supernatants were dialysed to equilibrium against [  $^3\text{H}$  ] Folate in 0.17 M Tris-HCl buffer of pH 7.4 for 24 h at 37 °C with Triton X-100 1g/l added to both sides of the dialysis membrane (30). Radioactivity was determined as previously described (30).

**Enzyme linked immunosorbent assay (ELISA):** A previously described ELISA employing an anti-human milk FBP antibody (25) which specifically immunoreacts with FBP/FR alpha was used (27). ELISA with antibodies against FBP purified from cow's milk was also conducted for further testing of the specificity of the immunoreactive response in ELISA (28). FBPs purified from human and bovine milk were analyzed in ELISA to control the analytical performance of the assays (25,28).

**Gel filtration:** Gel filtration experiments on an Ultrogel AcA 44 column (LKB) were performed as previously described in detail (30). Briefly, the column ( 5.3 cm<sup>2</sup> x 96 cm ) was eluted at 5 °C with Tris-HCl buffer of pH 7.4 containing 1 g/l Triton X-100. The column was calibrated as previously reported (30).

**Indirect immunofluorescence:** To demonstrate human alpha FBP in neutrophil granulocytes we used ethanol fixed smears of normal peripheral blood leukocytes as substrate (31). The smears were incubated with 1.5 mg protein /ml solutions of rabbit anti human alpha FBP, anti human lactoferrin, and rabbit serum (control) for 30 min in a humid chamber. Each smear was individually washed with excess of PBS and all smears are submersed in the chamber in PBS for 10 min to remove non-specifically attached serum. After removal of all PBS from the slides, the submersion of slides in PBS was repeated for another 10 min. Excess PBS was removed around the circle of each slide, but a minute amount of PBS was left in the circle to avoid drying of the sample. Fluorescein isothiocyanate-labelled Ig fraction of swine anti-rabbit serum (DAKO F0205) diluted 1:25 in PBS was added as a drop onto the encircled cell smear of each slide. The slides were again incubated for 30 min at room temperature in a humid chamber. The washing procedures were repeated. A drop of a 2:1 glycerol/PBS mixture was then applied to each smear and a cover glass was applied. The smears were read directly in a fluorescence microscope.

To show the specificity we absorbed the rabbit anti-human FBP antibodies with excess of human FBP and repeated the immunofluorescence procedures. The lightening of the cytoplasm in the neutrophil granulocytes disappeared.

The specificity was further tested by replacing rabbit anti human FBP antibodies by rabbit anti bovine FBP antibodies or rabbit serum.

## RESULTS

### *alpha FBP/FR in erythrocytes*

Antibodies against alpha FBP from human milk immunoreacted with serial dilutions of hemolyzed erythrocytes (Fig1). To test the specificity of response we performed a parallel analysis in ELISA with antibodies against bovine milk FBP ( Fig1 inset). The analytical performance of the two ELISA methods were controlled by running serial dilutions of purified human and bovine milk FBP ( Fig 1, Fig 1 inset).

Immunoreactive FBP/FR in hemolyzed erythrocytes did not bind [<sup>3</sup>H] folate in equilibrium dialysis experiments.

### *FBP in leukocytes*

The concentration of functional FBP, in terms of maximum binding of [<sup>3</sup>H] folate in equilibrium dialysis experiments, was determined in leukocyte lysates obtained from three healthy subjects. The FBP concentration in leukocyte lysate showed a great interindividual variation: one was < 0.01, one 3.8 and one 5.0 nmol/l.

Gel filtration of Triton X-100 solubilized leukocyte lysates containing 3.8 and < 0.01 nmol/l FBP were performed after preincubation with [<sup>3</sup>H]folate. The former sample exhibited a single peak of radioactivity at 29 kDa, whereas no peak was observed in the latter sample (Fig 2). No micellar aggregates of GPI-tailed FR appeared at 100 kDa.

### *alpha FBP/FR in neutrophil granulocytes*

ELISA with antibodies against human milk alpha FBP was performed to identify alpha FR/FPB in neutrophil granulocytes.

Serial dilution curves (two-fold initial dilution) of the three leukocyte lysates in Fig 2 showed a weak positive response in ELISA. The response was rather similar in the three samples despite widely different concentrations of functional FBP suggesting that alphaFR/FPB contributed very little – or most likely not at all to the total amount of functional FBP. The analytical performance of the ELISA was tested by running serial dilutions of purified human milk FBP (Fig 2).

Indirect immunofluorescence with antibodies against human milk alpha FBP identified the alpha isoform of FR/FPB in neutrophil granulocytes subjected to ethanol fixation (31). The positive immunostaining in Fig 3a exhibited a characteristic pattern involving the nuclei or perinuclear zone

of neutrophils. This type of pattern is supposed to be due to redistribution of cytoplasmic components to anionic components of the nucleus upon removal of lipids from granule membranes by ethanol fixation (31). Indirect immunofluorescence with antibodies against another cytoplasmic protein in neutrophils, lactoferrin, showed a very similar pattern of immunostaining (Fig 3b). No immunostaining was seen in Fig 3c with rabbit serum (control).

The specificity of the present anti-alpha FBP antibody was documented as follows.

Immunostaining disappeared when the indirect immunofluorescence procedure was repeated after absorption of the antibody with excess of human milk FBP (data not shown).

The typical immunostaining pattern disappeared when antibodies against bovine milk FBP were used (data not shown).

No immunostaining of beta FR on the surface of neutrophil granulocytes (14 -16, 22) .

### ***Correlation between functional FBP in serum and leukocyte lysate***

Matched data for serum and leukocyte concentrations of functional FBP from previous studies (19,20) were re-analysed in order to test a hypothesized relationship between the FBPs in granulocytes and plasma.

A weak positive correlation (statistically significant) between the concentrations of functional FBP in serum and leukocyte lysate was found in 15 healthy subjects with  $> 0.5 \text{ nmol FBP} / 10^9$  leukocytes (Fig 4).

### ***Functional FBP and alpha FBP in serum***

The concentration of [ $^3\text{H}$ ] folate bound FBP (functional FBP) was determined by equilibrium dialysis in pooled sera from pregnant women ( FBP, 0.73 nmol/l ), blood donors ( FBP, 1.10 nmol/l ), and umbilical cord blood ( FBP, 0.71 nmol/l). The pooled sera were analysed in ELISA with antibodies against human milk alpha FBP to screen for the presence of alpha FBP (Fig5).

Serial dilution curves of the three types of pooled sera showed weak positive responses in ELISA suggesting the presence of alpha FBP in serum. No response was seen when a parallel ELISA with antibodies against bovine milk FBP was performed to test the specificity (data not shown). The analytical performance of the ELISA was controlled by running serial dilutions of purified human milk FBP (Fig 5).

The gel filtration experiments in Fig 6 were performed to estimate the molecular size of functional FBP and alpha FBP in serum. A Triton X-100 solubilized pool of sera from blood donors was



preincubated with [<sup>3</sup>H] folate to identify functional FBP. The elution profile revealed a major peak (400 cpm) at a position of 29 kDa similar to that in leukocyte lysates as well as a small (60 - 80 cpm) broad and shoulder-like peak from 70-120 kDa.

The elution profile of alpha FBP determined in ELISA with antibodies against human milk alpha FBP contained a small peak at 43 kDa continuing into a broad peak with a maximum height (OD 0.6) at >120 kDa (void volume). No alpha FBP was found at a position of 29 kDa where the major peak of functional FBP had its maximum height (Fig 6).

## DISCUSSION

The most important finding herein was the identification of alpha FBP in human blood cells and plasma/serum.

A high-affinity FBP ( $K = 10^{-11} \text{ M}^{-1}$ ) anchored to surface membranes of mature human erythrocytes by a hydrophobic residue was first identified by Antony et al (12,13). Incubation of erythrocytes with trypsin resulted in the release of soluble hydrophilic FBP to the external medium (12,13). Indirect evidence suggested that erythrocyte FR is an alpha isoform: It immunoreacts with antibodies against human placental FR (12), which represents a mixture of alpha and beta isoforms (21), and erythrocyte membranes do not show immunostaining with antibodies against FR beta (22). Our ELISA data in Fig1 obtained with antibodies specifically directed against alpha FBP (26,27) provide direct evidence for the presence of FR alpha in mature erythrocytes.

In spite of removal of endogenous folate, free as well as receptor bound, by repeated washes at low pH, Antony et al. (12) who used a radiofolate preparation with a high specific activity only succeeded in identifying two molecules of functional FBP/cell in reticulocyte-rich young cells. A progressive up to seven-fold loss of functional FBP was observed as erythrocytes aged (12). No folate was released after boiling of the washed erythrocyte membranes indicating a complete removal of endogenous folate (12). Hence, the extremely small number of functional receptors explains why we failed to detect any binding of our low-specific activity [<sup>3</sup>H] folate to hemolysates despite repeated washes and prolonged dialysis to remove endogenous folate..

The functional role of erythrocyte FR is not clear, but the addition of anti-placental FR antiserum induced a decrease of intracellular folate and megaloblastic changes during in vitro erythropoiesis (32). Mature erythrocytes contain polyglutamated forms of folate suggesting that folate is taken up

at a more immature stage of erythrocyte development since the enzyme polyglutamate synthetase is only present in immature cells (12). Human hemoglobin has been identified as a low-affinity/high capacity binding protein for folate (29), and binding of folate polyglutamates to hemoglobin has also been reported (33).

Several studies have focussed on the presence of different isoforms of FBP in mature neutrophil granulocytes.

A FR beta has thus been identified on the surface of mature neutrophils and subpopulations of CD 34 positive cells in the myelomonocytic lineage by use of immunostaining and flow cytometry with anti- human FR beta antibodies (14-16, 22 ). No FR alpha is present on the surface membrane as evidenced by flow cytometry with monoclonal antibodies against FR alpha (14) as well as by our indirect immunofluorescence data (Fig 3). The FR beta had a variant GPI membrane anchor insensitive to PI-PLC but cleaved by nitrous acid (22). The FR beta in mature normal granulocytes remained non-functional in spite of acid washing to remove endogenous folate , whereas it was active in acute myeloid leukaemia cells with 0.72 pmol folate bound per  $10^6$  cells (22). The physiological role of FR beta expressed during neutrophil maturation is obscure, but in CD 34 positive cells it may serve a function that is distinct from its role in folate uptake, i.e. signal transduction and cell adhesion (16). The FR beta in myeloid leukemia cells is up-regulated 20-fold at the level of mRNA by all-trans retinoic acid ( 15) meaning that the receptor could be a promising target for therapeutic intervention, e. g. with folate conjugated cytotoxins, due to its selective up-regulation in leukemic cells (14, 22).

Besides the FR beta on the surface, neutrophil granulocytes contain a cytoplasmic functional high-affinity ( $K_d = < 1$  nM ) FBP only present in detectable amounts in a minority of healthy subjects ( 17-20). It was originally found in women who were pregnant or taking oral contraceptives (18), but the present data (Fig 2) as well as other studies reveal great inter- individual variations in concentration of functional FBP in both males and females (19,20), and clustering of persons with high concentration levels in certain families (34). The protein seems to be located in the matrix of secondary neutrophil granules, and just like lactoferrin released therefrom during degranulation induced by high lithium concentrations (35-38). It is supposed to be identical to the secretory FBP gamma isoform which is expressed in hematopoietic tissue and lacks a hydrophobic GPI residue (23,24). The appearance of a single peak of radioactivity at 29 kDa in the gel filtration profile of leukocyte lysate (Fig 2) is consistent with a gamma isoform possessing no GPI-tail.

The ELISA data in Fig 2 showed virtually similar concentrations of FBP alpha in three samples of leukocyte lysate despite widely different concentrations of functional FBP. Hence, FBP/FR alpha seems to contribute very little, most likely not at all, to the total content of functional FBP in the granulocyte. Indirect immunofluorescence data (Fig 3) revealed that FR alpha just like lactoferrin is located to a cytoplasmic structure in neutrophils. The exact nature of this structure is difficult to establish since the ethanol fixation procedure disrupts cytoplasmic membranes. A putative locus could be secretory vesicles since their membranes contain several GPI-linked proteins (39,40). One may argue that subpopulations of hematopoietic stem and progenitor cells do not contain FR alpha mRNA (14). This finding, however, does not exclude the presence of quiescent FR alpha mRNA having been or becoming active at other stages of differentiation.

At least two isoforms of FBP seem to be present in human serum.

A functional high-affinity FBP with a molecular size of 29 kDa similar to that of functional FBP in leukocytes (Fig 2 and 6). Data in Fig 4 strongly suggest that this FBP mainly derives from neutrophil granules (17-20).

An alpha isoform of FR/FBP that contributes very little to the total concentration of functional FBP in serum (Fig 5 and 6). It appears as a minor peak at 43 kDa continuing into a broad immunoreactive peak reaching its maximum in void volume (> 120 kDa). This isoform is present in serum at a low concentration as judged from its weak immuno-response, and could represent fragments of FR alpha from decomposed erythrocytes and neutrophils.

To summarize, the present report and other studies have revealed a rather complex expression pattern of FBP/FR isoforms in human blood cells. FR alpha on reticulocyte/erythrocyte membranes seems to mediate folate uptake and

to be of great importance for the erythropoiesis. The functional FR beta on membranes of leukemic granulocytes has been utilized for therapeutic targeting while its non-functional counterpart on membranes of normal neutrophil granulocytes could be a putative signal protein. At this point it is difficult to assign a biological/functional role to the cytoplasmic FR/FBP alpha identified in neutrophil granulocytes.

The biological role of the high-affinity FBP in serum is still unresolved. Like other soluble nutrient and vitamin binding proteins in serum, e.g. lactoferrin and B<sub>12</sub> vitamin binding protein which are released during degranulation of neutrophil granulocytes (35-38), FBP may exert bacteriostatic effects by depriving bacteria of endogenous folates (24). Binding to FBP protected tetrahydrofolate, the endogenous form of folate in pig serum, from biological degradation, whereas

low-affinity binding to albumin did not (41). So serum FBP may ascertain transport of intact folates to target organs/tissues.

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## LEGENDS

**Fig 1** The FBP alpha isoform in haemolysates identified by ELISA (OD, optical density) with antibodies directed against FBP alpha from human milk. Erythrocyte samples from five healthy subjects were analyzed in serial dilutions (each symbol represents haemolysate from a single individual). Analytical performance of the ELISA methods controlled by running serial dilutions of purified human milk FBP, 1.0 nM (filled circles).

Inset: A parallel ELISA of haemolysate performed with antibodies against bovine milk FBP to test the specificity of anti human milk FBP antibodies. Analytical performance of the ELISA controlled by running serial dilutions of purified bovine milk FBP, 1.0 nM (filled circles).

**Fig 2** The FBP alpha isoform in leukocyte lysate. Leukocyte lysates containing 3.8 nmol (circles), 5.0 nmol (squares), and <0.01 nmol (diamonds) [<sup>3</sup>H] folate bound (functional) FBP / 10<sup>9</sup> cells were sampled from three healthy persons, and analyzed serially diluted (two-fold initial dilution) in ELISA with antibodies against human milk alpha FBP.

Abscissa, concentration of [<sup>3</sup>H]folate bound FBP.

Ordinate, OD in ELISA.

Analytical performance of the ELISA controlled by running serial dilutions of purified human milk FBP, 1.0 nM (filled circles).

Inset: Gel filtration profile of functional FBP in leukocyte lysates. Leukocyte samples containing 3.8 nmol (circles) and <0.01 nmol (triangles down) [<sup>3</sup>H] folate bound FBP / 10<sup>9</sup> cells (cf above) were preincubated with 10 nmol/l [<sup>3</sup>H]folate in Tris HCl buffer of pH 7.4 containing Triton X- 100 1 g/l before column application.

Ordinate, cpm. Abscissa, elution volume.

**Fig 3** Indirect immunofluorescence for identification of cytoplasmic alpha FBP in neutrophil granulocytes.

Typical immunostaining patterns with primary antibodies against human milk alpha FBP (a), and another cytoplasmic protein, lactoferrin (b) Rabbit serum as control (c). All solutions containing 1.5 mg protein/ml.

Immunofluorescence disappeared when anti human milk FBP antibodies were preabsorbed with excess of human milk FBP. The immunostaining pattern disappeared when antibodies against human milk FBP were replaced with antibodies against bovine milk FBP (data not shown).

**Fig 4** Correlation between concentrations of [<sup>3</sup>H] folate bound FBP in serum (> 0.5 nmol/l FBP) and leukocyte lysate (abscissa) in 15 healthy individuals.

Correlation coefficient 0.644 ; p< 0.01 ( Spearman Rank Correlation). Data taken from references (19,20).

**Fig 5** The FBP alpha isoform in serum. Concentration of [<sup>3</sup>H] folate bound FBP determined in pooled sera from pregnant women (triangles up), umbilical cord blood (triangles down), and blood donors (diamonds) by equilibrium dialysis. Serial dilutions of the pooled sera analyzed in ELISA with antibodies against human milk alpha FBP.

Abscissa, concentration of [<sup>3</sup>H]folate bound FBP.

Ordinate, OD in ELISA.

Analytical performance of the ELISA controlled by running serial dilutions of purified human milk FBP (filled circles).

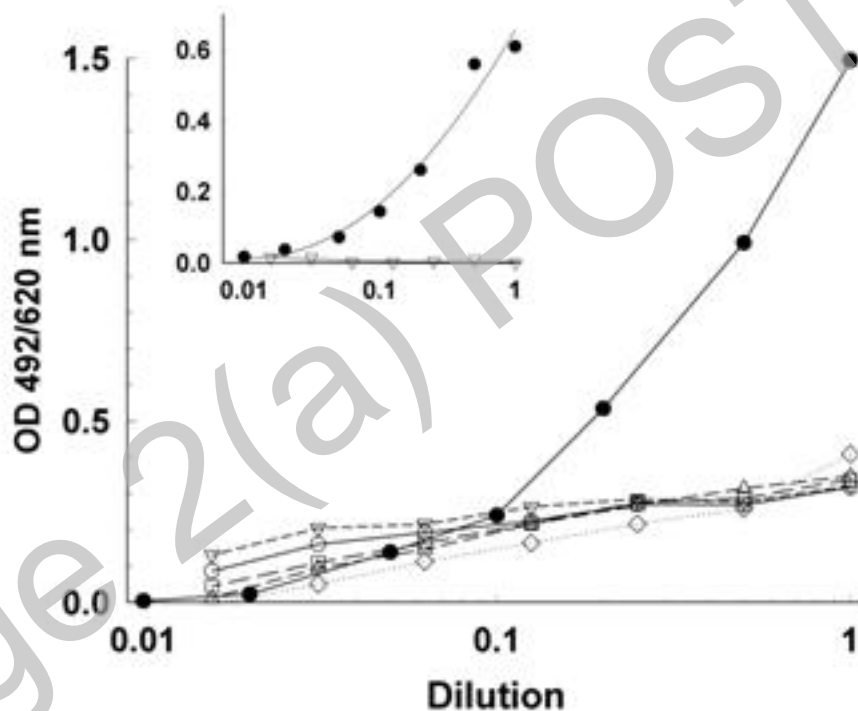
A parallel ELISA of serum was performed with antibodies against bovine milk FBP to test the specificity of antibodies against human milk FBP.

**Fig 6** Gel filtration profile of FBP isoforms in pooled sera from blood donors. Pooled sera were preincubated with 10 nmol/l [<sup>3</sup>H] folate prior to column application.

Left ordinate, cpm in effluent fractions (filled circles).

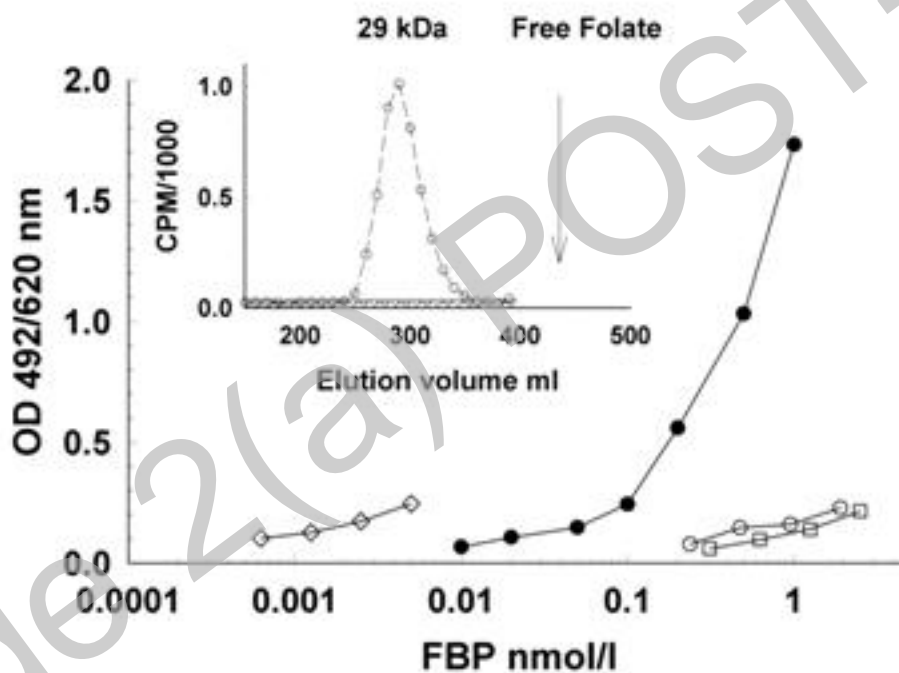
Right ordinate, OD measurements in ELISA on effluent fractions (open circles).

# Fig 1



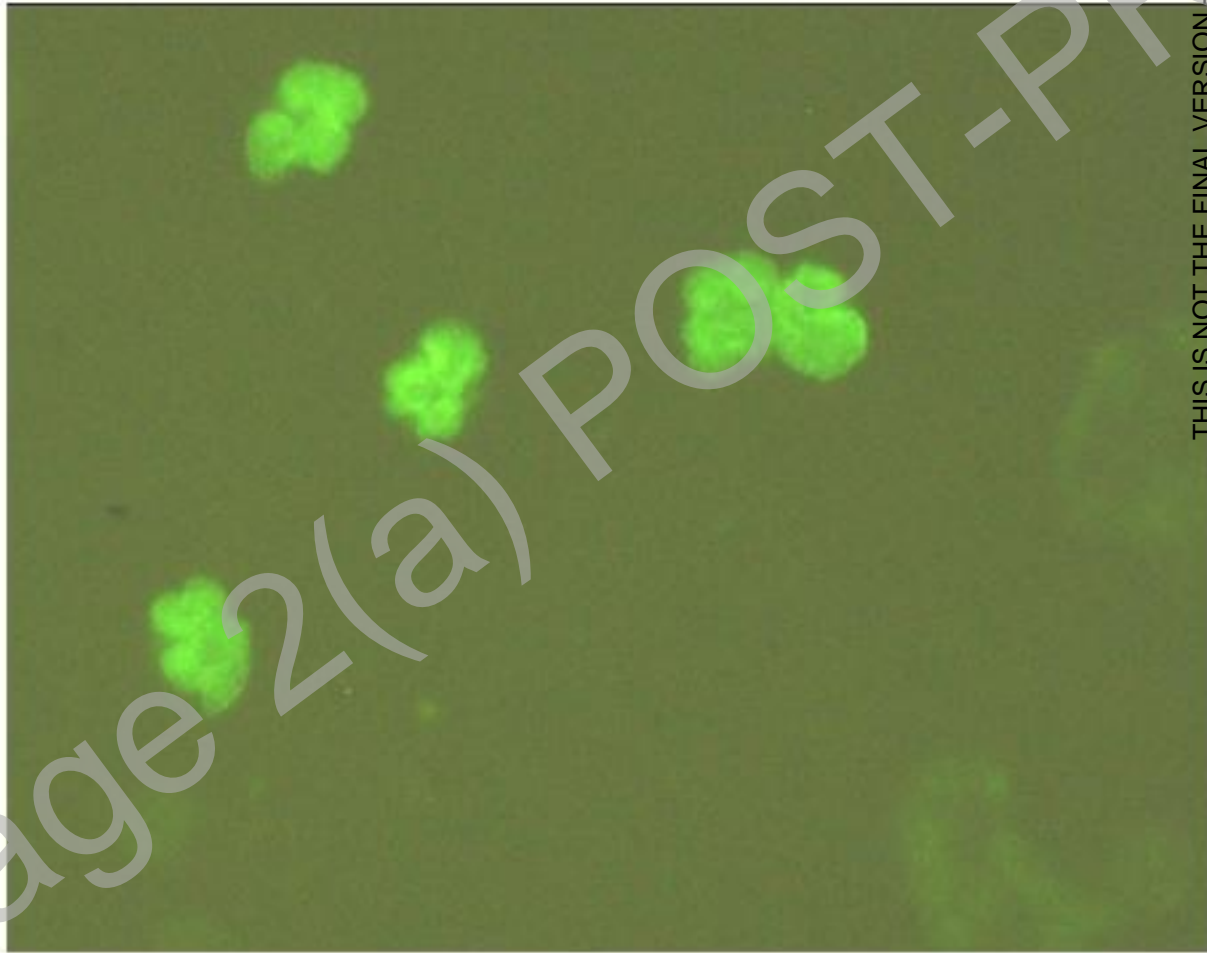
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# Fig 2



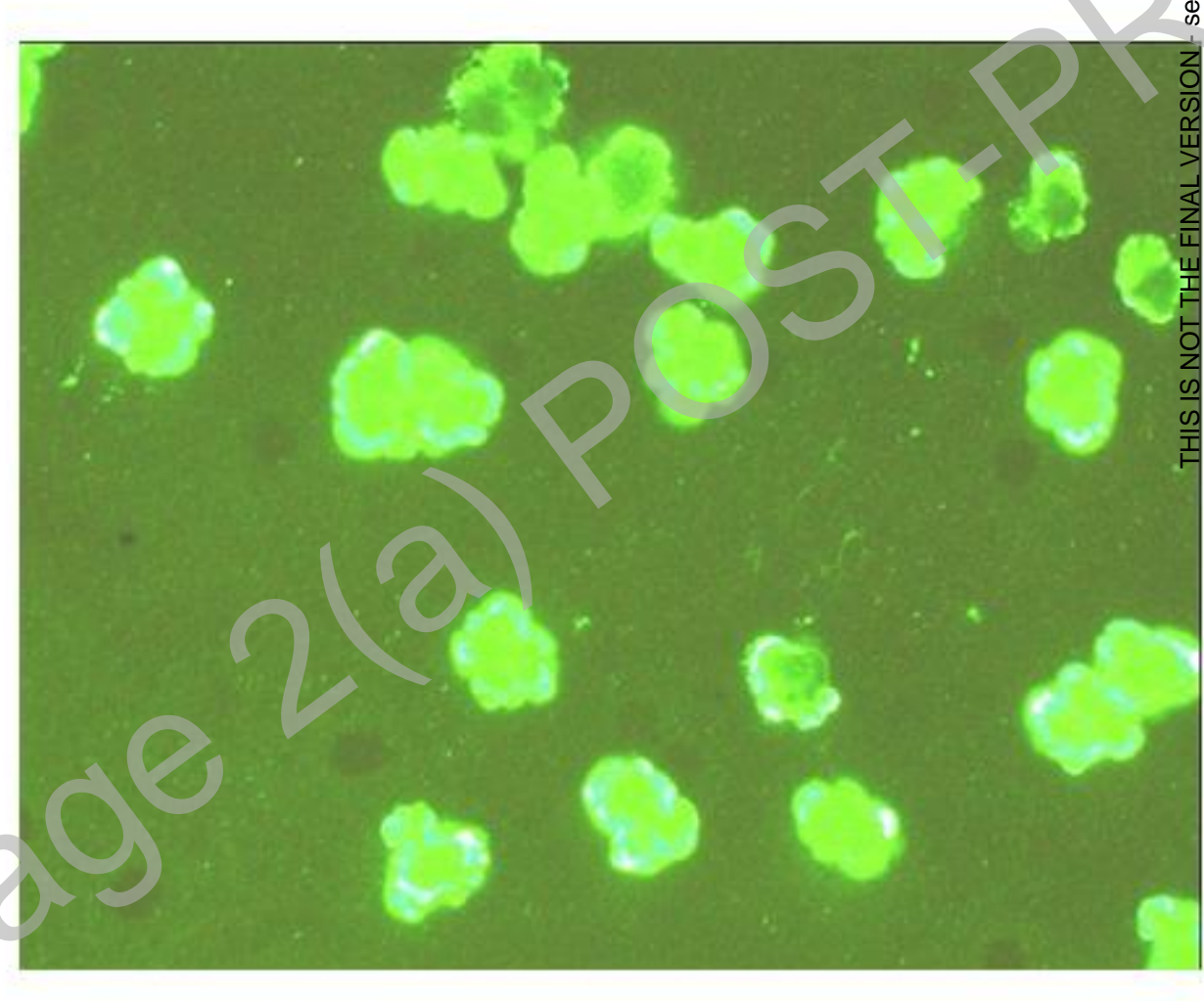
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# Fig 3A



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# Fig 3B



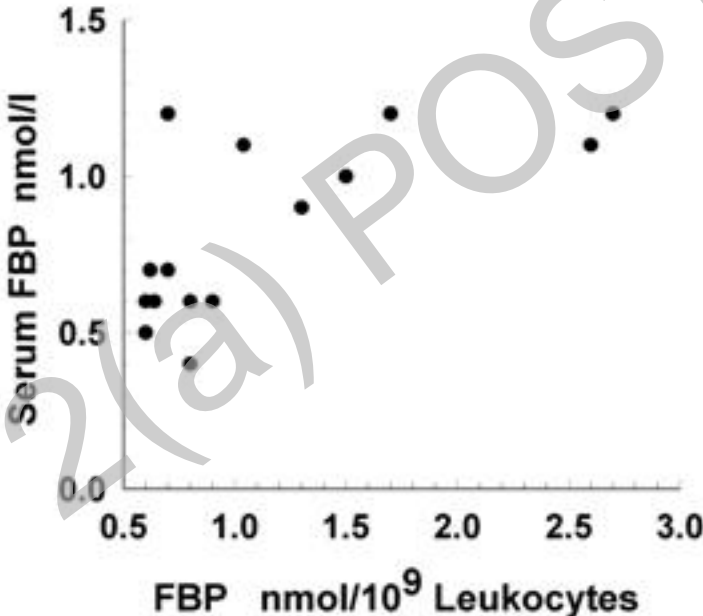
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# Fig 3C



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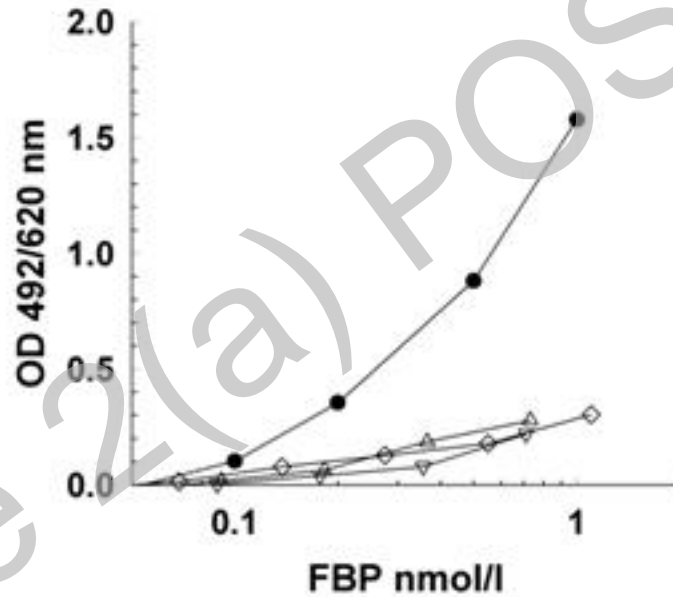
# Fig 4



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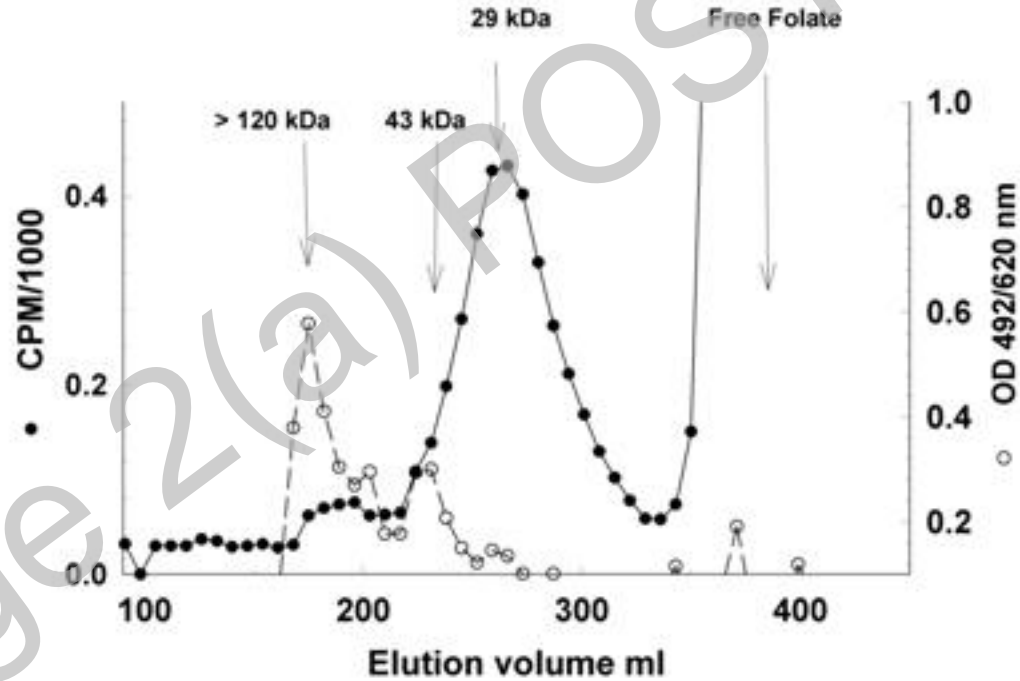


# Fig 5



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# Fig 6



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