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Antibody to folic acid: increased specificity and sensitivity in ELISA by using ε-aminocaproic acid modified BSA as the carrier protein

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Abstract

For raising high titre and specific antibody to haptens or drugs, ε-aminocaproic acid modified bovine serum albumin (ε-ACA-BSA) was prepared for use as a carrier protein. Folic acid (FA) was coupled to ε-ACA-BSA, Imj.BSA and BSA for raising antibodies in rabbits. Enhancement of FA immunogenicity with FA-ACA-BSA was observed. Apart from determination of titre by indirect ELISA, dose-response behaviour and specificity of these antisera were also compared. FA-ACA-BSA antibody showed high sensitivity and specificity. Using this antibody, an ELISA method for the determination of FA was developed. The study provides a simple approach to raise highly specific and high titre antibody against small molecules.

Keywords: Folic acid; ε-Aminocaproic acid modified bovine serum albumin; ELISA; Antibody titer

1. Introduction

Folic acid (pteroylglutamic acid, FA) is an essential vitamin required for formation of normal red blood cells. The deficiency of this vitamin causes nutritional anemia (Herbert, 1977). Development of a sensitive immunoassay for FA was hampered by non-availability of high titre antibody. Immunization with FA conjugates of proteins, synthetic polypeptides and methylated bovine serum albumin was reported to yield antibodies of low titre and specificity (Jaton and Unger-Waron, 1967; Rubenstein and Little, 1970; Da Costa and Rothenberg, 1971; Handel, 1981).

The antibody response in animals immunized with hapten carrier conjugates is often influenced by the nature of the carrier molecule. It has also been shown that the carrier not only influences the amount of anti-hapten antibody but also the type and avidity of the immunoglobulin produced

(Siskind et al., 1966; Butler and Beiser, 1973). The presence of a multiatom spacer between the hapten and the carrier macromolecule in the conjugate is reported to yield antibodies of higher specificity and affinity (Castro and Prieto, 1975; Pratt, 1978).

In the present communication, we report the preparation of highly specific antibody against FA by using ε-aminocaproic acid modified BSA as the carrier protein.

2. Materials and methods

2.1. Materials

Flat bottomed polystyrene microtitre plates (Maxisorp) and 8-channel microplate washer were from Nunc, Denmark. The automatic microtitre plate reader was from Bio-Rad Laboratories, Richmond, USA. Bovine serum albumin (cat. no. 2153), horseradish peroxidase (type VI), polyoxyethylenesorbitan monolaurate (Tween 20), dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3′-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), ε-aminocaproic acid, FA (pteroylglutamic acid), 5-CH_3-THF, 5-CHO-THF, pteroic acid, DHF, THF, pterine, *p*-aminobenzoic acid (PABA), formiminoglutamic acid (FIGLU), methotrexate, L-ascorbic acid (sodium salt), *N*-hydroxysuccinimide, trinitrobenzene sulfonic acid (TNBS), 3,3′, 5,5′-tetramethylbenzidine (TMB), Freund's adjuvant (complete and incomplete) and goat anti-rabbit IgG-peroxidase conjugate were purchased from Sigma, St. Louis, USA. Imject bovine serum albumin (Imj.BSA) was obtained from Pierce, Rockford, USA. Sephadex G-50 and Sepharose 4B were from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals and buffer salts were of analytical grade.

2.2. Buffers

Coating buffer was glycine-HCl (50 mmol/l, pH 2.3) and sodium phosphate (50 mmol/l, pH 7.6). Washing buffer was sodium potassium phosphate (50 mmol/l, pH 7.6) containing Tween 20 (0.5 ml/l). Post-coating buffer was sodium phosphate (50 mmol/l, pH 7.6) containing BSA (5 g/l) and thimerosal (0.1 g/l). Incubation buffer was sodium phosphate (50 mmol/l, pH 7.5 containing per litre, 150 mmol of NaCl, 1 g of BSA and 0.1 g of thimerosal). Enzyme assay buffer was sodium acetate/citric acid (100 mmol/l, pH 3.95).

2.3. Preparation of protein conjugates

ε-Aminocaproic acid modified bovine serum albumin (ε-ACA-BSA)

To a stirred solution of BSA (500 mg) in 10 ml of deionized water, ε-aminocaproic acid (1 g) and EDC (300 mg) was added. The reaction was carried out at room temperature for 4 h. After the reaction, the mixture was dialyzed extensively against phosphate buffer (10 mmol/l, pH 7.5). The product was lyophilized and further purified by passing through a column of Sephadex G-50 (1.5 × 40 cm) using phosphate buffer, 10 mmol/l, pH 7.5 as mobile phase. Protein fractions were pooled, lyophilized and stored at 4° C. Analysis of ε-ACA-BSA with TNBS method (Habeeb, 1966) showed no change in the number of amino groups compared to normal BSA.

FA-protein conjugates.

FA was covalently coupled to ε-ACA-BSA by the activated ester method as described by Mattox et al. (1979) using 25 mg of FA, 10 mg of *N*-hydroxysuccinimide, 23 mg of DCC and 100 mg of ε-ACA-BSA. The conjugate was purified by extensive dialysis against sodium phosphate buffer (10 mmol/l, pH 7.5) followed by chromatography over Sephadex G-50. Fractions with a constant ratio of absorbance at 340:280 nm were pooled and lyophilized (Fig. 1). The number of moles of FA bound per mole of modified BSA was approximately 20 as estimated by TNBS method (Habeeb, 1966).

FA-Imj.BSA and FA-BSA conjugates were also prepared in a similar way and the degree of conjugation of FA/protein ratio was found to be 18 and 19 respectively.

FA-ovalbumin conjugate was prepared by direct coupling of FA to ovalbumin by EDC using 50 mg of FA, 100 mg of EDC and 200 mg of ovalbumin at pH 8.5.

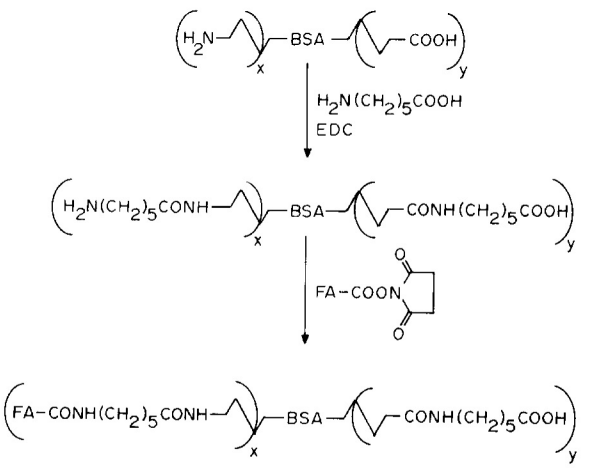

Fig. 1. Scheme for the preparation of ε-aminocaproic acid modified BSA and folic acid-aminocaproic acid-BSA conjugate (details in materials and methods section).

FA-peroxidase conjugate (FA-HRP)

Conjugation of FA to horseradish peroxidase was achieved by activated ester method described above. The product was purified through extensive dialysis followed by chromatography over Sephadex G-50. The molar ratio of FA to protein in the enzyme conjugate, as determined from the ratio of absorbence at 363 and 403 nm was 5.0. The working solution consisted of a 50-fold dilution of the conjugate (final concentration 0.02 g/l) in incubation buffer.

2.4. Immunization procedure

Three New Zealand White rabbits were immunised with each of the three immunogens, namely, FC-ACA-BSA, FA-Imj.BSA and FA-BSA conjugates following the method described by Dawson et al. (1978). Briefly, 1 mg of FA-protein conjugate in 1 ml of isotonic saline was emulsified with 1 ml of Freund's complete adjuvant and injected subcutaneously in multiple sites at the back of each animal. Booster injections with the same amount of antigen in Freund's incomplete adjuvant were administered after 2 and 4 weeks and thereafter at monthly intervals. Blood was collected from each rabbit after the third to seventh booster injections. The antisera were purified by precipitation with ammonium sulphate (50% saturation) followed by dialysis against phosphate buffer saline (10 mmol/l, pH 7.5). Finally, they were passed through a BSA-Sepharose immunosorbent column to remove anti-BSA antibody.

2.5. Antibody titre determination

The purified antisera obtained at different time intervals from different rabbits after immunization with FA-ACA-BSA, FA-Imj.BSA and FA-BSA were tested for antibody titre by indirect ELISA. The optimal concentration of antigen to be used for coating microtitre plates was determined in preliminary experiments.

Microtitre plate was coated with FA-ovalbumin conjugate (0.5 μg/ml, 200 μl/well) in coating buffer at 4° C overnight. After washing, the plate was incubated with 200 μl/well of post-coating buffer for 3 h at room temperature. The plate was washed again and to each well, 150 μl of antiserum was added at a dilution of 1/5000 to 1/100 000 and incubated at room temperature for 2 h. After washing the plate, 150 μl of goat anti-rabbit IgG-HRP conjugate (diluted 1/1000) was added. After incubation at room temperature for 20 min, the plate was washed and 150 μl of substrate solution (0.75 mmol/l of TMB, 0.01% H_2O_2 in enzyme assay buffer) was added. After 15 min the reaction was terminated by adding 100 μl of 4 N H_2SO_4. The absorbance at 450 nm was determined and titres were calculated as the antiserum dilution that would give an absorbance of 1.0 under the conditions described above.

2.6. Assay of folic acid

Coating of microtitre plate

Anti-FA antibodies of highest titre obtained during the immunization were used. The antibody were treated with glycine-HCl buffer (pH 2.3) for 15 s and immediately diluted with coating buffer. Each well of a microtitre plate was coated with 200 μl of diluted antibody and incubated at 4° C overnight. The wells were washed and vacant sites were blocked with post-coating buffer (200 μl) for 3 h at 37° C.

Enzyme immunoassay

To each well of a coated plate, 50 μl of sample or standard and 100 μl of enzyme conjugate were added in duplicate and the plate was incubated for 2 h at room temperature. The wells were washed and 150 μl of TMB substrate solution was added and incubated in the dark at room temperature. After 30 min the enzymatic reaction was stopped by adding 100 μl of 4 N H_2SO_4 per well and the A_{450} was measured.

3. Results and discussion

BSA was modified by reaction of ε-aminocaproic acid in presence of EDC. ε-aminocaproic acid contains one free amino and one carboxyl group and conjugation of either of these groups with carboxylic or amino groups of BSA is expected to leave the number of free amino or carboxy groups of the protein unchanged. This was corroborated by amino group estimation in the modified BSA by TNBS method. To minimise cross-linking of BSA the conjugation reaction was performed in high dilution. Polyacrylamide gel electrophoresis demonstrated the absence of any significant amount of cross-linked BSA. The UV spectrum of this protein was superimposable to that of native BSA.

FA was coupled to ε-ACA-BSA, Imj.BSA and BSA by the activated ester method and the FA: protein ratios in the conjugates were found to be 20:1, 18:1 and 19:1 respectively. The UV spectra of the conjugates were similar and showed the characteristic absorption of FA at 363 nm.

To evaluate the relative efficiency of the three carrier proteins in eliciting anti-FA antibody, we measured the antibody titres during the immunisation period. As shown in Fig. 2, the titre of antisera was seen to rise upto 3 months and then fall after every booster injection. The antibody titre of sera from rabbits immunized with FA-ACA-BSA reached 70 000–90 000 and then falls after 6 months to 62 000–70 000. In contrast, when FA-Imj.BSA and FA-BSA was used the titre reached only upto 35 000–60 000 and falls rapidly after 6 months to 25 000–48 000. The mean absorbance obtained with different dilutions of the anti-FA antisera obtained 3 months after primary immunization with the three different immunogens is shown Fig. 3.

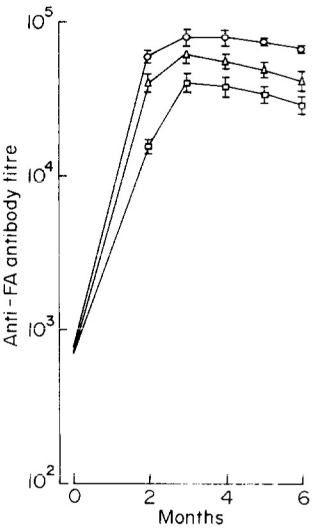

Fig. 2. Titre of folic acid antiserum drawn at various time intervals after immunization with FA-ACA-BSA (○); FA-Imj.BSA (△); and FA-BSA (□). Titres were calculated as the antiserum dilution that gave an OD of 1.0 under the indirect ELISA conditions. Values represent the mean and standard deviations of sera from three rabbits.

It may be pointed out that the antibody titre was measured using immobilized FA-ovalbumin conjugate which lacked the caproic acid spacer. It is expected that antibody against a hapten conjugate will have stronger binding towards a conjugate with spacer homology. In this case, the titre of antibody raised against two immunogens (FA-BSA, FA-Imj.BSA) without any spacer is much lower towards FA-ovalbumin than that raised against the heterologous immunogen-FA-ACA-BSA. This shows that, the titre of anti-FA-ACA-BSA antibody is intrinsically higher and is not due to bridge homology or heterology.

The three antisera were also tested for sensitivity and specificity by competitive ELISA. The optimised standard curves for the three sera using the same FA-HRP conjugate are shown in Fig. 4. The detection limit of FA and the range and slope of the three curve were quite different. The lower limit of detection (distinguishable from

blank by twice the SD) using anti-FA-ACA-BSA, anti-FA-Imj.BSA and anti-FA-BSA antibodies was 0.1, 1 and 10 pg/ well (2, 20 and 200 pg/ml) respectively corresponding to 95, 93 and 95% of the respective B_0. The corresponding FA concentration required to achieve 50% inhibition was 15, 70 and 120 pg/well (0.3, 1.4 and 2.4 ng/ml) respectively. The higher sensitivity and increased steepness of the standard curve obtained with antibody raised using ε-ACA-BSA may be anticipated due to the caproic acid induced bridging group heterology (Fujiwara et al., 1984; Manning, 1991).

Apart from dose-response characteristics, another important factor in assessing the suitability of an antisera is the specificity. A detailed cross-reactivity study was carried out with the same FA-HRP conjugate according to the procedure recommended by Abraham (1969). Comparative cross-reactivity of these antisera obtained for several folic acid derivatives is shown in Table 1.

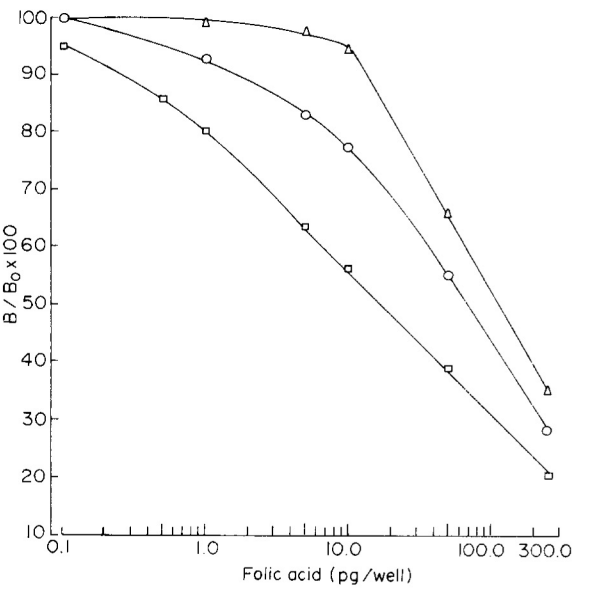

Fig. 4. Dose-response characteristics of anti-folic acid antisera obtained from three different immunogens: (a) FA-ACA-BSA (□); (b) FA-Imj.BSA (○); and (c) FA-BSA (△). Antiserum dilution used for coating in all cases were 1/8000. Dilution of enzyme conjugate were a and b, 1/8000; c, 1/4000.

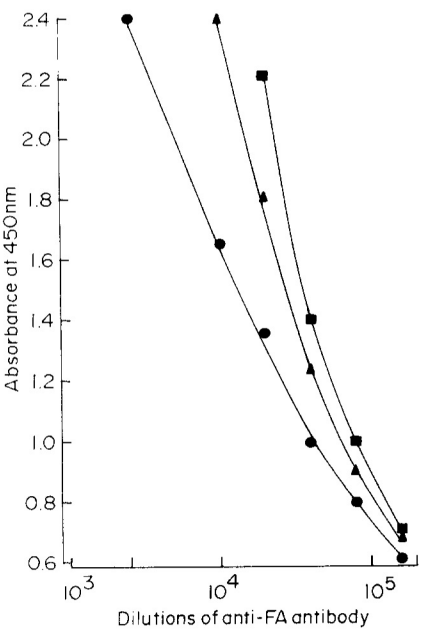

Fig. 3. Relationship between dilution and colorimetric signal level obtained by indirect ELISA. Antisera used were obtained 3 months after immunization with FA-ACA-BSA (■); FA-Imj.BSA (▲) and FA-BSA (●). The data represents the mean value from antisera of three different rabbits.

The results showed that cross-reactivity obtained with anti-FA-Imj.BSA and anti-FA-BSA antisera are very close. The anti-FA-ACA-BSA antibody showed high selectivity for FA and the cross-reactivity towards pteroic acid and DHF were remarkably low (8 and 10% respectively) compared to about 50% for other two antibodies. It may be pointed out that the structures of DHF and FA are very close – their only difference being the presence of a double bond between carbon atoms 7 and 8 in FA. The increased specificity with FA-ACA-BSA antibody is in accordance with the previously reported heterologous assays (Pratt, 1978). It may be noted that selectivity of folate binding protein used in folate assay for different folate analogues were reported to be 100%, 5 CH_3-THF; 127%, FA; 127%, DHF and 23%, 5-CHO-THF.

In conclusion, highly specific and high titre antibody against FA can be raised by using FA-ACA-BSA instead of FA-BSA as the carrier protein. The antibody can be used for estimation of

Table 1
Cross-reactivity of folate metabolites towards antibody raised against different folic acid immunogens

Metabolite	Cross-reactivity (%)		
	Antisera against [a]		
	FA-ACA-BSA	FA-Imj.BSA	FA-BSA
Folic acid	100	100	100
5-CH_3-THF	< 0.001	0.05	0.12
5-CHO-THF	< 0.001	0.04	0.25
DHF	10.0	50.0	40.0
THF	7.4	16.1	25.00
Pteroic acid	8.3	50.0	50.0
Pterine	0.01	2.0	1.3
PABA	< 0.001	< 0.001	< 0.001
FIGLU	< 0.001	< 0.001	< 0.001
Methotrexate	< 0.001	< 0.001	< 0.001

[a] Studies were done under identical conditions and repeated at least five times.

FA in multivitamin formulation and serum and a direct ELISA method for estimation of serum FA is described in the accompanying paper (Das Sarma et al., 1995). It is also expected that ε-ACA modified BSA may find wide application as a carrier protein for production of antibody against other small molecules.

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