The Isolation of Antibodies Specific for 5-Methyl-Cytidine-Bovine Serum Albumin

Sofia K. Mastronicolis *, Vassilios M. Kapoulas **, and Hans Kröger

Department of Biochemistry, Robert Koch-Institut des Bundesgesundheitsamtes, D-1000 Berlin 65, Bundesrepublik Deutschland

Z. Naturforsch. **36 c,** 459–463 (1981); received August 18, 1980/February 6, 1981

A-5-MeC Antibody, DEAE Sephadex, Precipitation Reaction, Antibody Quantitation, Immunodiffusion

By coupling 5-Methyl-cytidine to bovine serum albumin, a conjugate containing 18 mol of 5-Methyl-cytidine per mol of BSA was prepared. Antibodies to this conjugate were produced by immunization of rabbits. Specificity of the antibody was assesed by gel diffusion in agar containing excessive amounts of the carrier BSA. A slight cross-reactivity with cytidine was eliminated by adsorption on the cross-reacting antigen.

The isolation of 7 S immunoglobulin from the total globulin fraction was accomplished by chromatography on DEAE Sephadex-A-50, and a method for the rapid quantitation of the antibodies showed that 12.7% of the IgG protein are monospecific against 5-Methyl-cytidine bovine serum albumine.

Introduction

The production of immunochemical antigens, prepared by conjugation of naturally occuring nucleosides or nucleotides with a carrier protein, was developed since 1962 by Erlanger, Beiser *et al.* [1–10]. Originally, they prepared [1, 2] several purine or pyrimidine complexes with BSA or HSA by adaptation of the elegant method developed by Shaw *et al.* [11, 12] for synthesis of pyrimidines and N^1-pyrimidyl amino acids and for N-terminal amino acid analysis of proteins. The yield of the method, expressed as moles of purine or pyrimidine residues per mole of carrier protein, was 24 for purine-BSA, 27 for purine-HSA, 26 for 5-Ac-Ura-BSA, 9 for Ura-BSA and 19 for 5-Ac-Ura-HSA [2]. Later, they improved their method using the conjugation procedure of Khym and Cohn [13, 14], which increased the yields as follows: 29 for pU-BSA, 26 for G-BSA, 20 for C-BSA, 29 for pA-BSA [3]. By the same method Freeman *et al.* [5] obtained lower yields: 15 for A-BSA and 11 for G-BSA. Levine *et al.* [15] synthesized immunizing antigens (by the same method) with conjugates of 1-MeG-HSA, N^2-dimethyl guanosine-HSA, 7-MeG-HSA, N^2-MeG-HSA, and 5-MeC-HSA. Stolar *et al.* [16] prepared the conjugated complex of 1-b-D-ribofuranosyl-Thy with HSA. However, the yields are not given in these papers. Eichler *et al.* [17] prepared 5-nucleotide conjugates with BSA or RSA by the same method [3]. The yields of conjugation (expressed as above) were 26 for pG-BSA, 13 for G-BSA, 42 for pU-BSA, 36 for pC-BSA, 18 for pC-RSA, 18 for pA-RSA, 23 for pU-RSA, and 23 for pC-RSA. Finally Munns *et al.* [18] prepared N^6-MeA-BSA and 7-MeG-BSA and the yields were 17 and 51 correspondingly.

Another minor base of DNA is 5-methyl-cytosine, d-5-MepC is an almost universal although minor component of DNA in plants and animals [9]. It represents 4–7% of the bases in plant DNA [19], no more than 1.5% of the bases in mouse DNA [6] and even less in human DNA [20]. In the mouse, as in other mammals virtually all of the D-5-MepC is in the 5-MeCpG doublet [9], which is distributed non randomly in the DNA. Mouse satellite DNA contains about 3% of d-5-MeC in contrast to about 1.3% in the main band DNA, which is located at the centromeric end of nearly every chromosome. So we became interested in examining the possibility that

* Present address: Dept. of Food Chemistry, National University of Athens, 13 Navarinou street, Athens 114, Greece.

** Dept. of Biochemistry, School of Science, University of Ioannina, Ioannina, Greece.

Abbreviations: BSA (HSA, RSA)., bovine (human, rabbit) serum albumin; D. S. C., diluted saline citrate; C, cytidine; G, guanosine; A, adenosine; U, uridine; T, thymidine; 5-Ac-URA, 5-acetyl-uracil; 5-MeC, 5-methyl-cytidine; MeG, methylguanosine; d-5-MepC, deoxy-5-methyl-cytidylic acid; Thy, thymine; p-, phosphoric residue; 5-MeCpG, 5-methyl-cytidylphosphoguanosine.

Reprint requests to Dr. Sofia Mastronicolis, 3 Kassaveti Str., 816 Athens, Greece.

0341-0382/81/0500-0459 $ 01.00/0

antibodies (mono) specific for this minor component of DNA could directly interact with pure DNA.

The research described herein represents our initial findings regarding a) the conjugation of 5-MeC to BSA and the calculation of the degree of substitution b) the specificity of the antibodies elicited (in response to the above antigen) and c) the purification and quantitation of the above antibody.

Materials and Methods

Materials and reagents

Chemicals and media used in this work were purchased: 5-MeC from SIGMA; Complete Freund's adjuvant from DIFCO; Natrium metaperiodate, cytidine, amidoblack, sodium diethylbarbiturate, diethylbarbituric acid (proanalysi) from MERCK; BSA (purified) from Behringwerke AG. The solutions which were used were the following: DSC: 1 ml of 0.15 M sodium citrate-$2H_2O$ in 1.5 M NaCl was diluted to 100 ml with water. Gel system 7.5%: to 5 ml of an aqueous solution of 15% acrylamide and 0.375 % N,N-methylenbisacrylamide, 2 ml barbital-Na/HCl buffer, pH 8.2 and 2 ml H_2O were added. Barbital-Na/HCl buffer 0.15 M, pH 8.2: 769 ml 2.06% sodium diethylbarbiturate plus 231 ml 0.1 M HCl. Agarose gel: 1 g agarose was dissolved in 50 ml water and diluted to 100 ml with 0.15 M barbital-Na/HCl buffer pH 8.2. Barbital buffer pH 8.6: 1.33 g sodium diethylbarbiturate, 0.18 g diethylbarbituric acid, 0.68 g sodium acetate-$3H_2O$ in 100 ml H_2O. Agar gel: 1 g agar was dissolved in 25 ml barbital buffer pH 8.6 and 75 ml H_2O were added. The solution was boiled for 5 min and 100 mg BSA were added before cooling (at a temperature of 45 °−50 °).

Preparation of antigen

A conjugate of 5-MeC and BSA was prepared according to the method of Erlanger and Beiser [3]. BSA conjugates were also prepared by the same procedure, starting with 100 mg each of pA, G and C.

Quantitative data of the conjugated proteins were obtained from the U.V. absorption spectra in conjunction with the biuret [21] or the Lowry methods [22].

Polyacrylamide gel electrophoresis: To 9 ml 7.5% gel system, 150 µl 10% ammonium persulfate and 15 µl N,N,N,N-tetramethylethylenediamine were added. Electrophoresis was then started at 4mA per tube (1 h) and then continued for 2.5 h at 6mA per tube. Staining was then carried out with Amidoblack.

Agar gel electrophoresis was carried out exactly as above but ammonium persulfate was not removed prior to the run.

Immunization

Two rabbits (age: 6 months, weight 3 Kg) were injected with 5-MeC-BSA (1 mg/ml saline), emulsified with an equal volume of adjuvant according to the following schedule: each animal was given a total of 4.2 ml of this emulsion, divided into 9 doses, one every three days. The first dose was 1.0 ml, of which 0.6 ml were injected into toe pads "T P" (partly intradermally "I D" and partly into other tissues beneath the skin) and 0.4 ml were injected "I D". Each of the next 8 doses was 0.4 ml injected "I D". On the 3rd, 4th and 5th day after final injection the animals were bled and then on the 7th day 60 ml of blood were collected from each animal. Forty days after the collection of antisera, each animal was immunized with 1.0 ml of the antigen-adjuvant emulsion, injected as previously in the initial dose (booster injection). Eight days later, 55 ml blood were collected from each animal. All antisera were kept at − 20 °C.

Purification of antibody

Removal of cross-specificity by adsorption on C-BSA: Since the antisera exhibited a slight cross-reaction with C-BSA, they were purified at a first stage by the addition of C-BSA. The mixture was allowed to stay at 4 °C overnight and the precipitate was removed by centrifugation at $1000 \times g$ for 20 min. The required amounts of C-BSA were determined in preliminary experiments with 80 µl aliquots of antisera mixed with increasing volumes of the antigen (40, 60, 80, 100 . . . 240 µg).

Ammonium sulfate fractionation and DEAE-Sephadex chromatography: Precipitation of globulin fraction was effected by the method of Voss *et al.* [23] modified as follows: To 25−30 ml of antisera an equal volume of 3.2 M ammonium sulfate were added dropwise with simultaneous magnetic stirring. The precipitated proteins were removed by centrifugation ($20000 \times g$ 15 min), redissoved in 25 ml of 2 M ammonium sulfate at 4 °C and after continuous

stirring for 30 min, again centrifuged as above. The final precipitate was dissolved in 20 ml saline and the residual ammonium sulfate was removed by dialysis against 0.1 M Tris-HCl buffer, pH 8.0 (2 days at 4 °C). The ammonium sulfate fractions from each animal (19 and 15.7 ml correspondingly after determination of the protein content (106.4 and 229.2 mg) were applied at the top of DEAE-Sephadex A-50 columns (0.5 × 30 cm and 2.5 × 24.1 cm). The starting buffer was Tris-HCl, 0.1 M, pH 8.0, the final buffer Tris-HCl, 0.3 M, pH 8.0. Flow rate was 20–30 ml/h, fractions 3 ml. Protein concentration in the effluent was determined by measuring the optical density at 280 nm. The fractions containing IgG were pooled, and concentrated to 8 ml by ultrafiltration with AMICON apparatus (membrane UM 20 E).

Characterization of antibody

The original antisera and the preparations obtained at the various steps of purification were tested by *double radial* diffusion in agar gel, (see legend of Fig. 3 for details). Further examination was carried out by *immunoelectrophoresis* on agar gel at 200 V for 1.2 h, using a Gelman immunoelectrophoresis apparatus.

Quantitative precipitin techniques: The equivalence zone was determined with a slight modification of the method of Averdunk and Busse [24] to ascertain the amount of the antibodies against 5-MeC-BSA. Serial dilutions of 5-MeC-BSA in saline were made, and 0.5 ml of antisera (diluted 1:20 in saline) after adsorption with C-BSA and DEAE-Sephadex were mixed with 0.1 ml from each of the dilutions (see legend of Fig. 4). The solutions obtained were incubated for 20 min at 37 °C and the turbidity, produced by the antigen-antibody reaction, was measured at 436 nm in an Eppendorf spectrophotometer at 26 °C. These data revealed that equivalence point corresponds to the solution of 30 µg 5-MeC-BSA/tube.

The quantity of antibodies in the above purified antisera was determined as follows: An aliquot of the antiserum was diluted (1 to 5) and 0.4 ml of this solution was mixed with 0.1 ml of the equivalence point dilution of 5-MeC-BSA. After incubation at 37 °C for 1 h, the precipitate was sedimented at 1700 × *g* (10 min, 4 °C) and the protein contents of the supernatant and of the original (unprecipitated) solution were determined by the Lowry assay method.

The quantity of 5-MeC-BSA antibodies was then calculated by substracting the two values (total protein minus protein of supernatant).

Results and Discussion

Characterization of the antigen

The absorption maxima of BSA and 5-MeC are close: 279 and 277 nm respectively. Therefore the examination of the UV-spectrum of 5-MeC-BSA (Fig. 1) gives no information about the extent of the conjugation. On the other hand, electrophoretic methods give useful information about the yield of conjugation and the purity of the conjugate, but not about the degree of substitution. In polyacrylamide gel electrophoresis the conjugate preparation migrated entirely onto the anode *i.e.* quite distant from the three distinct bands obtained from native BSA. This finding indicated that the conjugation had been successful and that the antigen was practically free of unreacted BSA. This conclusion was further supported by agar gel electrophoresis (Fig. 2), which revealed that the conjugate migrates slowly toward the anode as a diffuse band, in contrast to native BSA, migrating slower as a distinct band. However

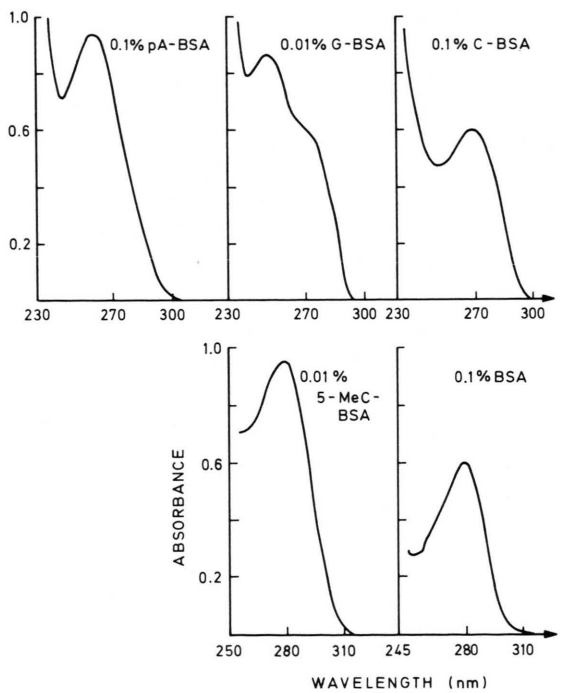

Fig. 1. Ultra violet absorbance spectra of various solutions of BSA-conjugates in D. S. C. obtained in a CARY spectrophotometer, light path 10 mm.

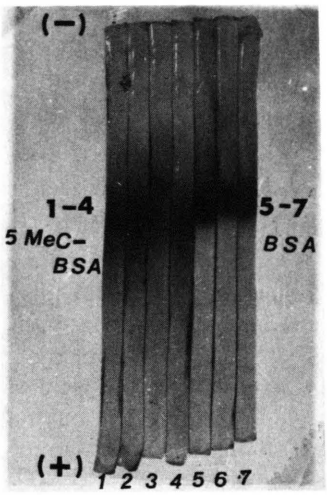

Fig. 2. Agar gel electrophoresis; The gel was agarose gel. To 30 µl of the sample solution (5-MeC-BSA 0.1% in 6% sucrose) one drop of 0.01% bromophenol blue was added.

for obvious reasons, the above results can not give any useful information about the degree of average substitution on the antigen. Therefore this value was calculated by an indirect method, employing the Biuret reaction [21] in combination with the measurement of the extinction coefficients of 5-MeC and of the conjugate at 277 nm.

Assuming a molecular weight of 66 000 for BSA, the molar extinction coefficients of BSA and of the conjugate are given by the expression: $E_{277} = 66\,000.$ A_{277}, where A_{277} is the optical density (at 277 nm) of BSA or conjugate solutions, containing 1 g protein/liter (or 1 mg/ml), as determined by the biuret assay method. The values A_{277} determined for BSA and for the conjugate were 0.66 and 3.04 respectively. Therefore:

$$E_{277\,(\mathrm{BSA})} = 66\,000 \times 0.660 = 44\,000$$
$$E_{277\,(\mathrm{Conjugate})} = 66\,000 \times 3.04 = 200\,640.$$

Under the same experimental conditions, the molar extinction coefficient of 5-MeC was found 8 900. The degree of substitution (D. S.) is the calculated by the formula:

$$\mathrm{D.\,S.} = E_{277\,(\mathrm{Conjugate})} - E_{277\,(\mathrm{BSA})}/8\,900 = 17.6.$$

This means that, approximately 18 mol of 5-MeC residues are bound to each mol of BSA in the prepared conjugate.

Characterization of antisera

Double radial immunodiffusion was used to examine the antisera of individual rabbits during immunization (3rd, 4th, and 5th days after the final injection). Since the immune response in all cases was positive the animals were bled finally on the 7th day and a total of 25 ml antiserum from each animal was collected. Fig. 3 (A_1 A_2) depicts typical immunodiffusion patterns from the first antiserum. These tests showed that in all cases, antibodies which strongly precipitate 5-MeC were present, whilst BSA-conjugates of the other 2 common nucleosides (G, pA) did not cross react. However a slight cross reaction with the C-BSA conjugate appeared.

Immunoelectrophoresis of anti-5-MeC antibodies have a precipitation pattern characteristic of only IgG. In this case, a precipitation band was formed only with 5-MeC, but not with C.

Purification of antisera and isolation IgG

After the adsorption of antisera with the required amound of C-BSA (approximately 25 ml of each antiserum required for clarification 2−3 ml of 0.8% C-BSA solution), the results from the double radial immunodiffusion (Fig. 3, B_1 and B_2) indicated that the antibodies were directed only against the 5-MeC hapten. After the isolation of 7S immunoglobulin from the total globulin fraction with ammonium sulfate and DEAE-Sephadex chromatography the final assays with double radial immunodiffusion indicated again the same results.

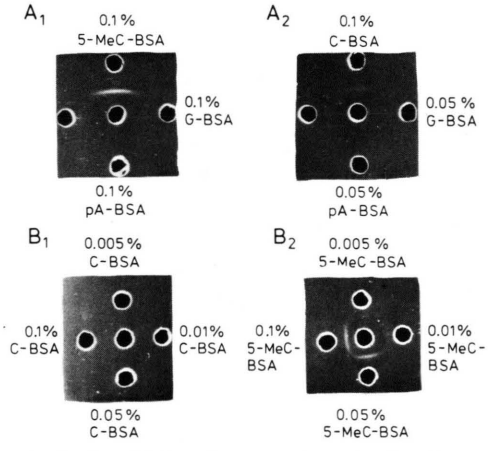

Fig. 3. Specificity of A-5-MeC antibodies. The center wells contained antibodies (10 µl) and the peripheral wells contained albumine-riboside conjugates (10 µl). The precipitation bands are seen in the agar gel (containing the carrier albumin) after double diffusion for 24 h at room temperature in a humid chamber. The photographs A_1, A_2 with crude A-5-MeC serum collected 7 days after the last administration of antigen. B_1, B_2 with the adsorbed antisera after the clarification with C-BSA solution.

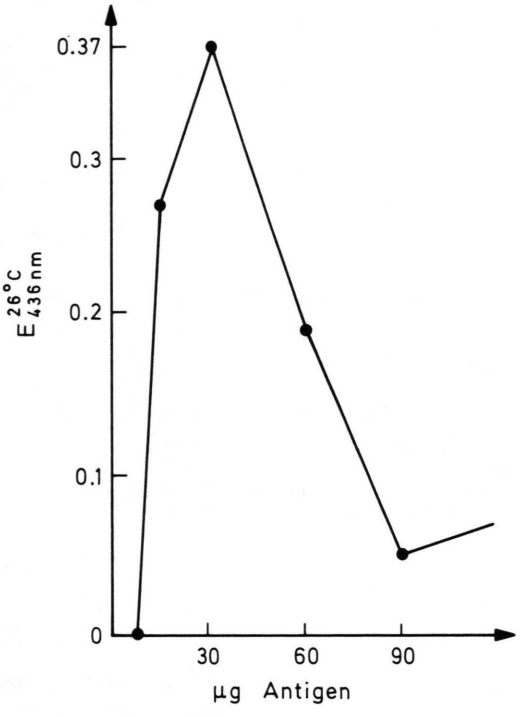

Fig. 4. The determination of equivalence zone by measurement of turbidity at 436 nm of the antibody elicited in response to 5-MeC-BSA. The points are solutions corresponding to 7.5, 15, 30, 60, 90 µg 5-MeC-BSA/tube (*e.g.* total volume in each tube 0.6 ml = 0.5 ml diluted 1:20 antisera and 0.1 ml from the next serial dilution: 75, 150, 300, 600, 900, 1200, 1500 µg 5-MeC-BSA/ml saline).

Estimation of antibody elicited in response to 5-MeC-BSA

Estimates of the amount of total A-5-MeC-BSA antibodies yielded values of approximately 1.27 mg of antibody per ml of adsorbed antisera. That means 12.7% of its proteins are IgG monospecific against 5-MeC-BSA (values represent the mean of triplicate assays).

Of major importance in this investigation has been the exploration of conditions of immunization in order to achieve monospecific antibody preparations of the highest possible concentration.

In this respect the adsorption of cross-reacting antibodies on C-BSA proved to be quite effective for the purification of the antiserum collected 7 days after the last administration of antigen.

In contrast, purification of the booster antiserum by the same technique resulted in an extensive coprecipitation of the desired antibodies along with the cross-reacting ones, yielding a purified antiserum of low concentration.

Acknowledgements

We acknowledge the helpful discussion with Dr. Peter Donner, Dr. R. Averdunk, Bjorn Wenzel, and Edgar Werner.

This work was supported by Deutsche Forschungsgemeinschaft.

[1] V. P. Butler, S. M. Beiser, B. F. Erlanger, S. W. Tanenbaum, S. Cohen, and A. Bendich, Biochemistry **48,** 1597 (1962).
[2] S. W. Tanenbaum and S. M. Beiser, Biochemistry **49,** 662 (1963).
[3] B. F. Erlanger and S. M. Beiser, Biochemistry **52,** 68 (1964).
[4] S. M. Beiser and B. F. Erlanger, Cancer research **26,** 2012 (1966).
[5] M. V. R. Freeman, S. M. Beiser, B. F. Erlanger, and O. J. Miller, Exp Cell. Res. **69,** 345 (1971).
[6] B. F. Erlanger, D. Senitzer, O. J. Miller, and S. M. Beiser, Karolinska Symposia on Research Methods in Reproductive Endocrinology (5th symposium) **168,** 206 (1972).
[7] S. M. Beiser and B. F. Erlanger, Internat. Convocation Immunol. **10,** 106 (1973).
[8] B. W. Lubit, R. R. Schreck, O. J. Miller, and B. F. Erlanger, Exp. Cell Res. **89,** 426 (1974).
[9] O. J. Miller, W. Schnedl, J. Allen, and B. F. Erlanger, Nature **251,** 636 (1974).
[10] B. W. Lubit, T. D. Pham, O. J. Miller, and B. F. Erlanger, Cell **9,** 503 (1976).
[11] R. K. Ralph, G. Shaw, and R. N. Naylor, J. Chem. Soc. **78,** 1169 (1959).
[12] J. H. Dewar and G. Shaw, J. Chem. Soc. **60,** 3254 (1961)
[13] J. X. Khym and W. E. Cohn, The journal of Biological Chemistry **236,** PC 9 (1961).
[14] J. X. Khym, Biochemistry **2,** 344 (1963).
[15] L. Levine, H. Vunakis, and R. C. Gallo, Biochemistry **10,** 2009 (1971).
[16] W. L. Hughes, C. Mary, and B. D. Stolar, Anal. Biochem. **55,** 468 (1973).
[17] G. Eichler and G. D. Glitz, Biochim. Biophys. Acta **335,** 303 (1974).
[18] T. W. Munns, M. K. Liszewski, and H. Sims, Biochemistry **16,** 2163 (1977).
[19] H. S. Shapiro and E. Chargaff, Biochim. Biophys. Acta **39,** 68 (1960).
[20] P. D. Lawley, A. R. Crathorn, S. A. Shah, and B. A. Smith, Biochem. J. **128,** 133 (1972).
[21] H. Gräser, Biochemisches Praktikum p. 63, Vieweg F & Sohn. GmbH, Verlag, Braunschweig 1971.
[22] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193,** 265 (1951).
[23] H. Voss, G. Henneberg, R. Herrmann, H. Pichl, S. Schulte-overber, and H. Werner, Zbl. Bakt. Abt. I Orig. **205,** 249 (1967).
[24] R. Averdunk and V. Busse, Z. Klin. Chem. Klin. Biochem. **7,** 287 (1969).