Monospecific Antibody against 5-Methyl-Cytidine for the Structural Analysis of Nucleic Acids

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Anti 5-methyl-cytidine antibodies might be useful agents for the detection and localization of 5-methyl-cytidine of nucleic acids, but only if the antibodies recognize this nucleoside with sufficient specificity. A conjugate containing 18 moles of 5-methyl-cytidine per mole of BSA was prepared and antibodies directed against this nucleoside hapten were produced by immunization of rabbits (as determined by gel diffusion in agar containing excessive amounts of the carrier). A slight crossreaction of cytidine-BSA was eliminated by adsorption on the cross-reacting antigen. Further purification of the antibodies was effected 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-BSA. Hydrolysis of antibodies with insolubilized papain produced monovalent Fab fragments which were identified by SDS-Disk-electrophoresis. A two stage method for cross linking the immunoproteins to ferritin by glutaraldehyde was used. The isolation of immunoferritin conjugates by Bio-Gel A 1.5 m column chromatography is described. The identification of the effluents was made by glycerin density gradient ultracentrifugation. The results were visualized by electron microscopy after the treatment of immunoferritin conjugates with (methylated and unmethylated) denaturated DNA, fractionation on the glycerine density gradient, and the spreading by a modification of drop technique.

Introduction

The production of immunochemical antigenic conjugates of naturally occurring nucleosides or nucleotides with a carrier protein, for the application to problems of genetics and transcription, was developed since 1962 by Erlanger, Beiser *et al.* [1–10], Levine *et al.* [11], Stolar *et al.* [12], Eichler *et al.* [13], Munns *et al.* [14]. D-5-methyl cytidylic acid is an almost universal minor component of DNA in plants and animals [15]. It represents 4–7% of the bases in plant DNA, no more than 1.5% of the bases in mouse DNA and even less in human DNA [16]. In the mouse as in other mammals virtually all of the D-5-methyl cytidylic acid is in the 5-methyl-cytidyl-phosphoguanosine doublet [15], which is distributed non randomly in the DNA. Although antibodies specific to several minor bases of DNA (*e.g.* methyl cytosine, methyl guanosine etc.) have been reported, their direct interaction with pure, denaturated DNA has never been described in the literature. The present work describes a method for the production of antibodies specific against 5-methyl-cytidine and our attempts to test their direct interaction with denaturated methylated and unmethylated DNA.

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Experimental Methods

Materials and reagents

Chemicals and media used in this work were purchased: 5-MeC from Sigma; Complete Freund's adjuvant from Difco; The Papain, ENZYGEL from Papaya carica (from Boehringer Mannheim) contained 10% L (+)-cysteine and 90% carrier bound enzyme (specific activity 80 U/g); glutaraldehyde and Formvar from Serva; cytochrom *c* of horse heart (0.43% Fe, salt free) from Serva. The solutions used were the following: 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 complete cooling (temperature 45–50 °C). Diluted glutaraldehyde: solution 25%. Diluted saline citrate (DSC): 1 ml of 0.15 M sodium citrate in 1.5 M NaCl was diluted to 100 ml with water.

All concentrations were made by ultrafiltration on AMICON apparatus (membrane UM 20 E). Ultra violet absorbance spectra of various solutions obtained in CARY spectrophotometer, light path 10 mm.

Preparation of antigen

The periodate-oxidized 5-methyl-cytidine (5-MeC) conjugate with crystalline bovine serum albumine (BSA) was prepared according to Erlanger and Beiser [17]. Also BSA conjugates were prepared by the same procedure starting with 100 mg each of adenosine 5-monophosphate, guanosine and cytidine. The purity of the conjugate was examined by polyacrylamide gel and agar gel electrophoresis.

Immunization, purification and characterization of antibody

Two rabbits were injected with 5-MeC-BSA (1 mg/ml) emulsified with an equal volume of adjuvant (9 doses, one every three days). Blood was collected (60 ml) and all the antisera were kept at −20 °C. Purification at a first stage was made by the addition of the required amount of cytidine-BSA for removing the slight cross reactivity with cytidine-BSA (approximately 25 ml of crude antiserum required 2−3 ml of 0.8% cytidine-BSA solution). Further purification was effected with ammonium sulfate precipitation and with column chromatography (DEAE Sephadex A−50) by a slight modification of the method of Voss *et al.* [18, 20]. Characterization of antibody was made by double radial diffusion in agar gel, containing excessive amounts of carrier BSA. The quantitative determination of precipitating antibodies of antiserum was made with a slight modification of the method of Averdunk and Busse [19, 20].

Enzymatic hydrolysis of IgG globulins and characterization of Fab fragment by SDS-disk electrophoresis

1.76 ml of purified A-5-MeC antibodies solution (17 mg/ml in 0.3 M Tris-HCl pH 8.0) was dialyzed against 0.1 M sodium phosphate buffer pH 7.0 and hydrolyzed by papain ENZYGEL with a slight modification of the Porter's method [21] with the following schedule: After dialysis, EDTA was added to a final concentration of 0.002 M, and 75 mg papain ENZYGEL was suspended into it, followed by the addition of 0.1 M sodium phosphate buffer pH 7.0, to a final volume of 2 ml. This suspension was incubated at 37 °C for 16 h under stirring. The papain was removed by filtration through Whatman, glass fiber paper GF/C (2.5 cm). The filtrate was dialyzed against 0.01 M sodium acetate buffer, pH 5.5 and applied to a CM-Cellulose column (31 cm × 0.9 cm). The volume of mixing chamber was 240 ml. Buffers used on the column were 0.01 M sodium acetate buffer, pH 5.5 with gradient to 0.9 M sodium acetate buffer pH 5.5 (Flow rate 40 ml/h). The fractions containing both peak I and peak II (total volume 51.3 ml) were pooled (also the fraction of peak III, vol. 29 ml). Protein concentration in the effluents was estimated by reading the absorption at 280 and 260 nm. Then, they were concentrated by ultrafiltration, dialyzed against 0.05 M sodium phosphate buffer, pH 7.0 and refrigerated.

We have used the method of disk-electrophoresis in sodium dodecyl sulphate (SDS) of Laemmli [22] for the characterization of Fab fragments.

Gels containing 3% (stacking gel) and 4.8% acrylamide were prepared from a stock solution of 30% (W/W) acrylamide and 0.8% (w/w) of N′,N′-bis methylene acrylamide. The final concentrations in the separation gel were as follows: 0.375 M Tris-HCl (pH 8.3) and 0.1% SDS. The gels were polymerized chemically by the addition of 0.025% (w/v) of tetramethylethylenediamine (TEMED) and ammonium persulfate. Ten-cm gels were prepared in glass tubes of a total length of 15 cm and with an inside diameter of 0.15 cm. The stacking gels of 3% acrylamide (length 1 cm) contained 0.125 M Tris-HCl pH 6.8 and 0.1% SDS and were polymerized chemically in the same way as for the separating gel. The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS. The samples applied on the gel prepared by mixing 20 µl of the solution (approx. 16 µg Fab I and II, or Fc) with 60 µl of "final sample buffer" 0.05 M Tris-HCl pH 6.8, 2% SDS, 10% glycerin, 5% 2-mercaptoethanol and 0.001% bromophenol blue as the dye. Electrophoresis was started with a current of 15 mA per gel until the bromophenol blue marker reached the bottom of the 3% in acrylamide stacking gel, (about 3/4 h). Then it was continued with a current of 35 mA per gel until the marker reached the bottom of the separation gel. After the electrophoresis, the separation gels were placed in staining solution

(15% in MeOH, 9.2% in CH_3COOH and 0.04% in Coomassie brilliant) over night [23]. The gels were destained by electrophoresis and by diffusion with solvent circulation for 1 h (12 v) in the apparatus of Pharmacia "Destainer CD-4" (Destaining solvent 7% aqueous acetic acid).

Preparation and purification of immunoferritin conjugates

The two stage method of Otto *et al.* [24] was used for cross-linking immunoglobulin to ferritin (F) by glutaraldehyde (GA). In the first step, freshly diluted GA was added to 200 mg F in 0.05 M phosphate buffer, pH 6.8 – 7.0 to a final volume of 4.7 ml. This solution was incubated for 2 h at 37 °C and filtered through a Sephadex G-25 column (23 × 2.6 cm) to remove unreacted GA. The effluent F-GA (ferritin with glutaraldehyde) was concentrated to 3.4 ml by ultrafiltration. In the second step two samples were prepared, the sample A with Fab fragments (I and II) and the sample B with non hydrolyzed IgG, in a 1 : 5 (w/w) ratio of protein to F-GA. The final concentration of F-GA was 15 mg/ml. Both samples were allowed to stand for 24 h at 37 °C. At the end of conjugation, 0.01 M L-lysine was added. The unreacted F and the unreacted IgG and Fab molecules were separated from the conjugates by column chromatography as follows:

1 ml conjugate IgG-FGA (Sample B) was applied at the top of Bio-Gel A 1.5 m column (82 × 1.25 cm) at 4 °C, with 0.05 M phosphate buffer pH 7.2 (flow rate 7 ml/h, fractions 5 ml). After determination of the F concentration in the IgG-FGA fraction by spectrometry (at 440 nm) the solution was ultrafiltered to 3.2 ml and identified by density gradient ultracentrifugation (see Fig. 2 a).

2 ml conjugate Fab FGA (sample A) was applied first at the top of a Sephadex G-200 column (34.5 × 2.5 cm), and eluted as above (fractions 3 ml). The effluents of the first peak were ultrafiltered to 3.8 ml and further fractionation was effected by applying at the top of Bio-Gel A 1.5 m column (same conditions as for fractionation IgG-FGA), and the effluents of the first and second peaks were ultrafiltered to 1.5 and 2.6 ml respectively.

Denaturation of DNA

The available DNA samples were a) DNA (unmethylated) from *M. lysodeicticus* (L) and b) DNA (methylated) from rat liver (R) isolated by the method of Marmur [27] and Kirby [28] respectively. Stock solutions in DSC (L: 700 µg/ml, R: 550 µg/ml) were diluted 2 : 3, and subjected to heat denaturation by immersing in boiling water for 10 min, followed by cooling in ice bath. The above operations were carried out immediately before use in the ultracentrifugation experiments described below.

Ultracentrifugation

The density gradient rate centrifugation experiments were performent at 50 000 rpm near 4 °C in a Beckman model L5-65 rotor SW 65K. The samples (110 – 200 µl) were layered on the top of 5 ml 10 – 30% glycerol density gradient and were centrifugated for the required lengths of time (50 – 150 min). After the sedimentation of the macromolecules in separate zones the UV light diagrams were traced, by an ISCO model 640 density gradient fractionator (Dual beam UV visible optical unit model 1140).

DNA samples of the same chain length were obtained by ultracentrifugation, as above, for 105 min. Fractions of 0.5 ml were collected by the ISCO fractionator. The peak effluents (~ 1 ml) were diluted with an equal volume of water, the concentration was measured by 0 D_{260} and this solution was kept in refrigerator as the stock solution for coupling.

The diagrams showed that all the available species of DNA chains (methylated or unmethylated) had a M.W. slightly lower than that of the ferritin-labeled Fab or IgG molecules. Although longer DNA chains would be desirable, the available ones were sedimentable when attached with at least two antibody molecules per chain DNA.

Fractionation of the coupling product of denatured DNA with the immunoferritin conjugates

On the basis of the fact that about 1% of the bases of rat liver DNA are methylated, it may be calculated that 1 µg of R-DNA contains approx. 30 pmol of methylated bases, corresponding to $30 \times 500\,000 = 15 \times 16^6$ pg = 15µg of specific immunoferritin conjugate. Since in our preparation the specific conjugate corresponds to about 10%, we used 300 µg of IgG-FGA per µg of R-DNA (twice the amount required). Each time, solutions containing 1 µg denatured R or L-DNA (of the same chain length) was added to 150 µl (~ 300 µg) solutions

Fab-FGA or IgG-FGA and made to a final volume of 233 µl with 0.1 M EDTA. After incubation at 37 °C for 30 min, they were cooled in ice bath and were fractionated by density gradient centrifugation for 50 min. Marker solution was 15 µl Fab-FGA plus 215 µl D.S.C. The solution above the yellow zone was removed very carefully with a 0.6 mm diameter needle; the rest of the solution was pipetted out leaving at the bottom the last 16 – 100 µl of liquid (corresponding to 0.5 – 1.0 µg DNA). The latter were used for spreading.

Electron microscopy

Grids of the type Polaron Equipment G-400 size 2.3 mm coated with 1.2% formvar in $CHCl_3$, were coated with a thin layer of carbon with the aid of a freeze fracturing and etching accessory type EDWARDS 306. Samples were prepared by a modification of the drop technique [25, 26] using teflon coated dishes. 15 – 200 µl aliquots of DNA fraction (0.5 – 1 µg DNA) from glycerol gradient ultracentrifugation were added to a solution of 0.2 M ammonium acetate containing 20 µl 0.01% Cytochrome *c* in 0.2 M ammonium acetate and 5 µl 35% formaldehyde and adjusted to a final volume of 1 ml (0.2 M in ammonium acetate concentration). This mixture was kept at room temperature for 20 – 25 min. The samples were picked up on the carbon-coated grids, washed in ethyl alcohol, dried and rotary shadowed with platinum in the EDWARDS 306 aparratus. A Siemens Elmiskop 101 was used for electron microscopy.

Results and Discussion

Characterization of the antigen and of the antibody

The substitution of BSA by 5-MeC hapten was 18 mol of 5-MeC per molecule of the conjugate [20].

Double radial immunodiffusion was used for crude antisera, for the adsorbed antisera (with the cytidine-BSA solution) and for the isolated, after DEAE-sephadex chromatography, A-5-MeC-BSA antibodies. The results indicated that the antibodies were directed only against the 5-MeC hapten. After the fractionation of IgG fractions chromatographically, the quantitation of antibodies [20] showed that 12.7% of the proteins of the IgG fraction are monospecific against 5-MeC-BSA. This corresponds to 1.27 mg monospecific IgG per ml.

Fig. 1. Disk-polyacrylamide gel electrophoresis (in sodium dodecylsulphate) of Fab I and II and Fc fragments of the antibody specific for 5-MeC-BSA; a, marker proteins: Trypsin inhibitor (T.I) M_r: 21 000 and BSA M_r: 68 000; b, pooled effluents of peaks I and II from CM-cellulose column fractionation of hydrolysed antibodies; c, pooled effluents of peak III; d, mixture of b and c.

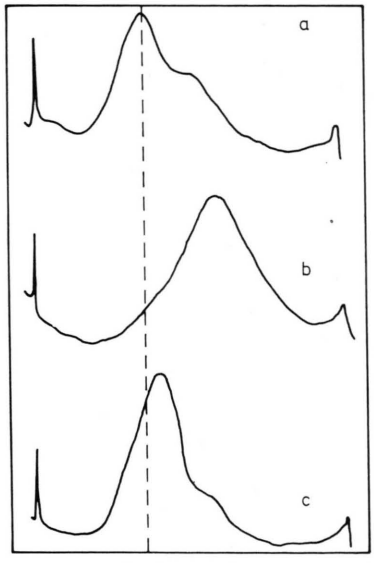

Fig. 2. UV-Sedimentation patterns of immunoferritin conjugates eluted from Bio-gel A 1.5 m columns. The samples were ultra centrifuged in a 10 – 30% glycerol density gradient and fractionated from an ISCO model 640 fractionator; a, IgG-FGA (second peak effluents from the column chromatography of Ferritin-labeled IgG on Bio-gel A 1.5 m); b and c, first and second peak effluents (respectively) from the rechromatography on Bio-gel A 1.5 m of the mixture of aggregate and Ferritin labeled Fab antibody, obtained from Sephadex G-200 fractionation; sedimentation is from left to right.

Production and characterization of Fab fragments.

Since intact antibodies precipitated DNA, it was necessary to convert the antibodies to a monovalent form for use as blocking agents in DNA sequence analysis. For this reason, they were cleaved into Fab Fragments by papain treatment followed by fractionation on a CM-cellulose column with a gradient of increasing salt concentrations. Three components appeared, corresponding to Porter's peaks for Fab I, Fab II and Fc, although in our case peak I appeared very close to the peak II. For this reason, they were pooled and further tested by SDS-electrophoresis.

We know that fragments Fab I and II each of M_r 50 000 resulting from papain hydrolysis (Porter method) of γ-globulins, are extremely similar in chemical, physical and biological properties, whereas the third fraction, Fc (M_r 80 000) is degraded to fractions of lower molecular weight by this method. On the other hand, SDS with its known capability to break down proteins into their individual polypeptide subunits, separates the two chains of Fab I and II into fragments of molecular weight 20 000 – 25 000. Our tests showed that the pooled Fab I and II migrate as a single band with a molecular weight similar to that of trypsin inhibitor (M_r 21 000) which was used as marker protein (see Fig. 1).

Preparation, purification and indentification of immunoferritin conjugates

In order to visualize by electron microscopy the reaction between antigen and antibody, the electron-dense metalloprotein ferritin was used by the aid of GA. By Bio-Gel A column chromatography, the im-

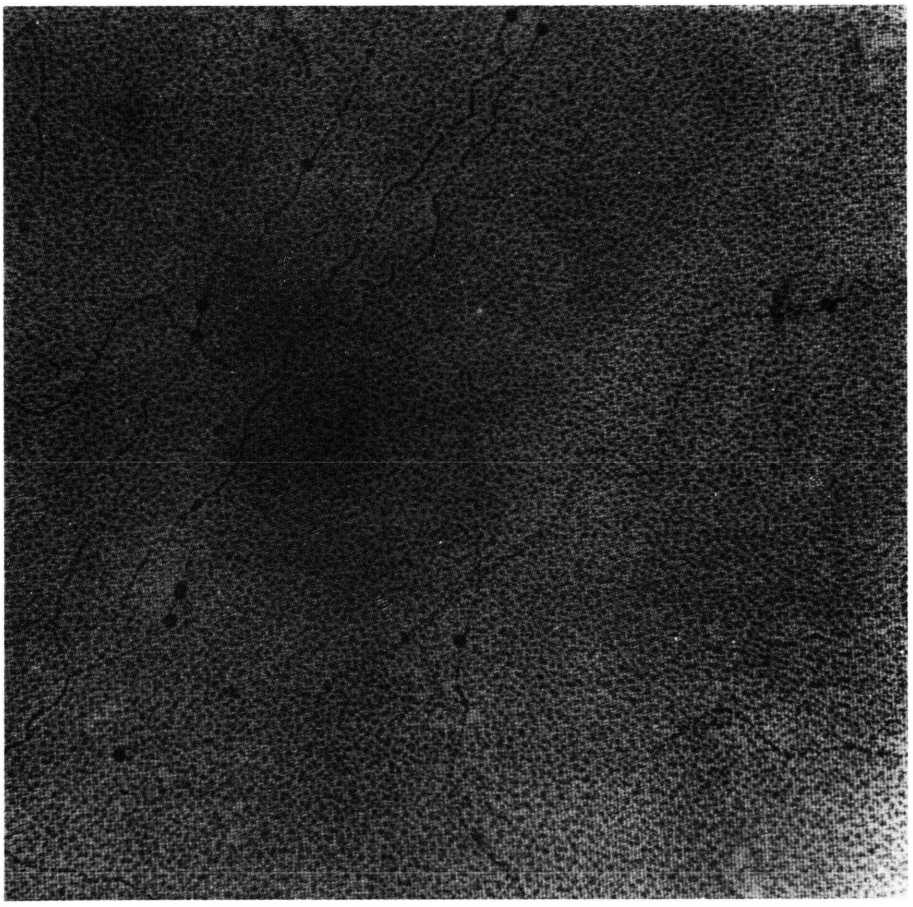

Fig. 3. 0.5 µg heat denatured rat liver DNA pretreated with 300 µg Fab-FGA after spreading and electron microscopy (Siemens Elmiskop 101, magnification of 40 000×).

munoferritin conjugate IgG-FGA was separated from the unreacted ferritin (aggregate) and from the free IgG molecules.

The Fab-FGA conjugate was not fractionated successfully on the Sephadex G-200 column because the aggregate was not separated from the conjugate and this would prevent fractionation by density gradient ultracentrifugation of DNA. So the effluents of the first peak were fractionated by Bio-Gel A 1.5 m and samples 110 µl of the effluents of each peak, as well as of the IgG-FGA fraction, were layered on the top of glycerol density gradient (10 – 30%) and ultracentrifuged for 65 min (Fig. 2 b, c). The ultra violet light diagrams (from the Isco model 640 fractionator) showed that the fractionation was successful *i.e.* Fab-FGA was eluted in the second peak, since it had the same M_r as the molecule IgG-FGA.

Electron microscopy of the coupling products

The electron microscopical examination of the product of coupling of the unmethylated DNA (from *M. lysodeicticus*) with Fab-FGA showed that neither ferritin conjugate molecules, nor DNA chains had been sedimented after ultracentrifugation for 50 min. The same results were also found by using ferritin-labeled IgG instead of Fab-FGA.

In contrast, when methylated DNA (from rate liver) was coupled with Fab-FGA, several DNA – chains bound to electron-dense molecules of the ferritin – conjugate sedimented and appeared in the electron microscopical photographs (Fig. 3). Considering that the DNA chain length used was rather short, and that their average content in methylated bases is only 1%, the above results indicate that our monospecific A-5-MeC-BSA antibody is suitable for the recognition of 5-MeC on the DNA chains with absolute specificity.

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