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Coupled gel electrophoresis–agar diffusion method for the detection of tumor antigens

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Abstract

A gel electrophoretic method coupled with agar diffusion has been devised for detecting tumor antigens in human colon tissue. Separation of the antigens is achieved on duplicate electrophoretic gels. One gel is used for the location of the antigens by protein staining and the other gel is used for assaying of the antigenicity by agar diffusion against homologous antiserum. Analysis of perchloric acid extracts of colon tumors by this coupled method revealed the presence of carcinoembryonic antigen and two additional glycoprotein antigens. Analysis of KCl–HCl tumor extracts revealed two new tumor antigens.

1. Introduction

Glycoprotein antigens are commonly extracted from colon tumors with perchloric acid. The carcinoembryonic antigen (CEA) is perhaps the most thoroughly characterized tumor-associated antigen of this class. Initially CEA was reported to possess high specificity with homologous immune serum. This led to the expectation that the antigen would be a valuable tumor marker specific for adenocarcinoma of the colon [1]. However, further studies not always confirmed this high specificity [2,3]. CEA is a glycoprotein with a molecular mass of $1.5–2.0 \cdot 10^5$ [4,5], containing 35–50% carbohydrate by weight [6,7] and belonging to a family of immunologically related gene products [8–10]. The carbohydrate chains may be joined O-glycosidically [11] or N-glycosidically to the protein moiety [12]. Many members of the CEA family possess immunodeterminants in the protein moiety [13] but a few possess immunodeterminants in the carbohydrate moiety [14]. Antigens with carbohydrate structure have been successfully extracted with a KCl–HCl solution of pH 2 from bacterial cell walls [15,16]. Samples of normal and tumor colon tissue have now been extracted by both the perchloric and the KCl–HCl methods and the extracts so obtained have been examined for tumor antigens by the coupled method described in this report. Immune sera were prepared by immunization of rabbits with the tumor extracts and Fruend's adjuvant. The tumor antigens in the extracts were detected by the new method of gel electrophoresis coupled with agar diffusion. It was found that the perchloric acid extracts of colon tissue with adenocarcinoma contained CEA and two additional CEA-crossreacting antigens of the CEA family. The extracts prepared by the KCl–HCl method contained two new tumor antigens which were distinct from the above antigens and did not crossreact with these

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antigens. In extracts of normal colon tissue from the same individuals the coupled method did not detect the presence of any tumor antigens.

2. Experimental

2.1. Gel electrophoresis and agar diffusion

Polyacrylamide gel electrophoresis was used in the coupled assay and was essentially performed as described by Davis [17]. A water cooled electrophoresis chamber (Buchler Instrument) was used for the analysis. A 7.5% acrylamide (Bochringer Mannheim, Indianapolis, IN, USA)/2% N,N^{1}-methylene bisacrylic (bis) gel (Eastman Kodak, Rochester, NY, USA) was used for separating the antigens. Samples of 50–300 μg of the tissue preparations in a volume less than 200 μl were used. The stock buffer was 0.05 *M* Tris–0.4 *M* glycine buffer (pH 8.4). The chamber buffer was a 1:10 dilution of the above buffer. The tubes were 10 cm in length and 0.6 cm in diameter. Electrophoresis was conducted for 3 h at a constant current of 2.5 mA per gel (Beckman power supply). At completion of the electrophoresis the gels were forced out of the tubes using moderate water pressure. One set of gels was fixed for 30 min at 60°C in a solution of 9 g of sulfosalicylic acid and 30 g of trichloroacetic acid in 200 ml of water and stained with Coomassie blue for 30 min at 60°C to reveal protein components, utilizing a staining solution of 0.1 g of Coomassie brilliant blue G-250, 13 g of salicylic acid and 7.3 ml of perchloric acid in 100 ml of water. After the staining period the gels were destained in 5% acetic acid. The other set of gels was not stained.

To locate the antigens a coupled electrophoresis–agar diffusion procedure was used. In the latter procedure, duplicate samples of the antigens were subjected to identical electrophoresis conditions. One finished gel was stained for protein and the other gel was embedded in fluid agar in a petri dish (diameter of 9 cm). When the agarose solidified, the plate was placed on moist filter paper in a larger petri dish (diameter of 15 cm). A trough was cut in the agarose about 2 cm from the polyacrylamide gel. Samples of 0.1–0.3 ml of the immune serum were placed in the trough. Diffusion was allowed to proceed at room temperature and the development of precipitin bands was noted. The plates with bands were photographed.

2.2. Tissue specimens

Colon specimens from individuals undergoing surgery were collected by Dr. E.B. Rosenberg (Mount Sinai Hospital, University of Miami, Miami, FL, USA) and shipped to this laboratory in a frozen state. Histological examination at the time of collection revealed well-differentiated adenocarcinoma in all diseased specimens. These specimens were collected under contract with the National Cancer Institute and their use for the current study was approved by the NCI project director. Specimens of normal colon tissue from the same individuals were also obtained. All tissue specimens were kept frozen at −70°C until used for experimental purposes.

2.3. Extraction with perchloric acid

Tumor specimens which were used for extractions were dissected as cleanly as possible to remove adhering normal tissue. The samples from six individuals were combined and cut into small pieces yielding a sample of approximately 50 g. The sample was divided into two equal parts. One part was extracted by the perchloric acid method [4] and the other part by a KCl–HCL method [16] as described below. In the perchloric acid method the tissue was homogenized in 2 volumes of 1.2 *M* perchloric acid in a Waring blender for 10 min. Following the homogenization, the liquid suspension was separated from the large tissue fragment by decantation. The resulting suspension was then stirred vigorously at room temperature for 6 h and finally subjected to centrifugation at 4300 *g* for 15 min. The precipitate was discarded and the supernatant saved for further treatment. The lipid particles in the supernatant were removed by recentrifugation at 20 200 *g* for 15 min and the top layer obtained by suction was discarded. The

remaining aqueous layer was dialyzed against distilled water at 4°C for 40 h and then passed through a 0.22-μm Millipore filter. The clear filtrate was lyophilized to dryness. A similar extraction of normal tissue was performed. The yield of extract was 0.1 g from the tumor tissue and 0.05 g from normal tissue from the same individuals.

2.4. Extraction with KCl–HCl

The other part of the sliced tissue was extracted with KCl–HCl solution. The extraction solvent was 0.05 *M* KCl–0.01 *M* HCl (pH 2.0). The pH of the solution was checked with a Corning pH meter and adjusted if necessary. The tissue was homogenized in two volumes of KCl–HCl solution in a Waring blender for 15 min. The homogenate was subjected to an identical treatment as described for the perchloric acid extraction. An extract of normal colon tissue was also prepared by the KCl–HCl method. The yields of lyophilized products from normal and tumor tissue obtained by the KCl–HCl extraction procedure were comparable to the yields obtained by the perchloric acid method.

2.5. Antisera with specificity for tumor antigens

Samples of 15 mg of the tumor antigen preparations, designated E_1 for the perchloric acid extract and E_2 for the KCl–HCl extract, were dissolved separately in 3 ml of 0.01 *M* phosphate buffer (pH 7.2) and saline. The resulting solutions were mixed with an equal volume of complete Freund's adjuvant. The suspension of each preparation (1 ml) was used to immunize different rabbits intraperitoneally at multi sites of the back of the neck weekly for a period of 5 weeks [18]. Blood specimens were collected after the last immunization and in subsequent weeks and antisera samples were prepared in the usual manner. Microzone electrophoretic patterns showed a significant increase in the gamma globulin in the serum samples after the second immunization cycle. Capilliary precipitin tests with the antisera and the corresponding antigen preparations were positive.

2.6. Agar diffusion and hapten inhibition

Agar diffusion tests were performed with the antigens and antiserum samples by the Ouchterlony method [19]. The results with the tissue extracts showed that specific antibodies were produced on immunization of rabbits with tumor extracts. Antibodies directed against the tumor extracts were not produced on immunization with the normal tissue extracts.

Micro inhibition, agar diffusion and quantitative precipitin techniques were used to test for hapten inhibition and nature of the immunodeterminants of the antigens [20]. L-Fucose was found to act as a strong inhibitor of the reaction of the KCl–HCl extract with homologous antiserum. At concentrations of 80 mg of L-fucose per ml of test solution 86% inhibition was obtained while at concentrations of 20 mg per ml only 19% inhibition was obtained. Mannose, galactose and acetyl glucosamine at the same concentrations as fucose yielded much lower values. The perchloric extract gave little inhibition with all the carbohydrates. Methylation and GC–MS analysis [21–23] revealed that many of the fucose units were terminal units of the carbohydrate moiety of the KCl–HCl extractable antigens. GC–MS analysis was done in a Carlo Erba GC equipped with a 60-m J and W Scientific capillary column coupled to a Kratos MS 25 spectrometer and using electron-impact ionization.

2.7. Periodate oxidation

Samples of the tumor extracts, CEA and the glycoproteins, glucose oxidase and lactosyl-BSA, were subjected to periodate oxidation and the products were tested for antigenicity [24]. Samples of 300 μg of the preparations were dissolved in 0.1 ml of water and mixed with 0.1 ml of 0.04 *M* sodium periodate of pH 4.5. Oxidation was allowed to proceed for 6 h in the dark at room temperature. An agar diffusion plate was prepared with the non-oxidized and the periodate oxidized samples against the antiserum specific for each antigen preparation. Diffusion was allowed to proceed until precipitin bands were

formed and subsequently the plate was photographed.

2.8. Carbohydrate analysis

Samples of approximately 2 mg of the tumor preparations were hydrolyzed in 0.01 ml of 0.2 *M* hydrochloric acid for 2 h in a boiling water bath. Paper chromatographic analysis of the hydrolysates was done by a standard method [25]. A solvent system of *n*-butyl alcohol–pyridine–water (6:4:3, v/v) and the multiple ascent technique were used. The carbohydrates were located by staining with $AgNO_3$ and KOH reagents. The carbohydrate hydrolytic products yielded brown colored spots and were identified by R_F values. The following monosaccharides with their apparent R_F values shown in brackets were present: fucose (0.62), mannose (0.56), galactose (0.51) and glucosamine (0.45). The hydrolysates also yielded a positive thiobarbituric acid reaction establishing the presence of sialic acid in the preparations [26]. These carbohydrate residues are typical of the carbohydrate moiety of CEA [4,13].

3. Results and discussion

The discovery in 1965 by Gold and Freedman [1] of CEA in human colon tumors and the projected use of the antigen as a tumor marker was an important advance in tumor immunology. The initially reported high specificity of the immune serum for this antigen led to great expectations and much research particularly for clinical utilization. However, additional results did not always confirm the high specificity initially reported [2,3]. Further, it has become apparent that CEA is a member of a family of crossreacting multigene products [27]. It has been found that the family of antigens is encoded by approximately 14 genes [28]. Progress in this area has been made with the isolation of a clone which contains the entire coding region of the CEA gene [29]. The detection of members of the CEA related family should be possible by analysis with the coupled method of electrophoresis and agar diffusion.

Some typical results of the analysis of extracts of colon tumors by the proposed coupled method are shown in Fig. 1. Comparable amounts of the extracts and CEA were used for the electro-

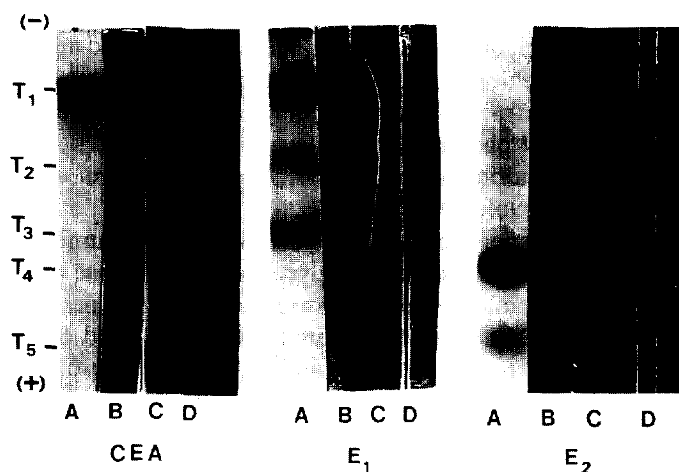

Fig. 1. Gel electrophoresis and agar diffusion pattern of colon tumor antigens: CEA = carcinoembryonic antigen, E_1 = colon tumor extracted with perchloric acid, E_2 = colon tumor extracted with KCl–HCl, A = stained gel, B = embedded gel, C = precipitin area, D = trough of serum and T_1–T_5 = location of tumor antigens in gels.

phoresis. Five distinct tumor antigens were extracted by the perchloric acid and the KCl–HCl solutions from diseased tissue of human colons. In Fig. 1, the CEA frame shows results with CEA (T_1) which was extracted with perchloric acid solution and purified by electrophoresis and gel filtration [4]. It can be seen from the figure that the CEA stains with protein stain yielding a single band and when tested by agar diffusion against tumor immune serum also yields a single antibody–antigen precipitin arc. Similar analysis was performed on an equivalent sample of the perchloric acid extract (E_1) from colon tumors. In addition to the positive CEA results, two other antigenic compounds were detected in the tumor extract. Agar diffusion of these compounds against homologous antiserum yielded three precipitin arcs, one corresponding in position to the precipitin arc of CEA (T_1). The other arcs are the result of the precipitin reaction of two additional tumor antigens (T_2, T_3) from the diseased colon with homologous antiserum. Extraction of the tumor tissue with KCl–HCl followed by the analysis of the extract revealed the presence of two new tumor antigens (T_4, T_5) which are different from those obtained by the perchloric acid extraction. The antigens of either group were not present in normal colon tissue in amounts that could be detected by the coupled method.

The antigenicity of CEA, the normal tissue extracts and the tumor extracts with the two types of immune sera were determined by the Ouchterlony agar diffusion method [19]. The results are shown in Fig. 2. CEA (C) reacts with serum 1 but not with serum 2 and extracts of normal tissue (N1 prepared by perchloric acid and N2 prepared by KCl–HCl) do not react with either serum. Therefore the antigens in the tumor extracts are produced during tumor development and are not present in extracts of normal tissues. Further it is noted that the perchloric acid extract (E_1) reacted with its homologous antiserum (S_1) and not with S_2 and the KCl–HCl extract (E_2) reacted only with the homologous antiserum (S_2). The latter results show that crossreactivity with the sera and the two types of antigenic extracts did not occur.

Fig. 3 shows some interesting effects of periodate oxidation on the tumor antigens, a glycoprotein and a glycoconjugate. It can be seen that on oxidation CEA, E_1 and G (glucose oxidase) retained their antigenicity. Since carbohydrate units are destroyed by oxidation, the carbohydrate units of these antigens are most likely not the immunodeterminant groups. The antigenicity is lost on periodate oxidation of the KCl–HCl tumor extract E_2 and L (lactosyl bovine serum albumin) demonstrating that the carbohydrate units are the immunodeterminants of these antigens.

That the new antigens detected in perchloric acid extracts of tumor tissue originated from proteolysis of CEA is unlikely for the following reasons. The electrophoretic pattern for the purified CEA (Fig. 1) consists of only one

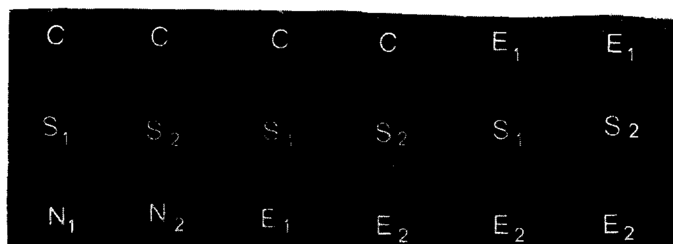

Fig. 2. Agar diffusion plate: C = CEA, N_1 = extract of normal colon tissue with perchloric acid, N_2 = extract of normal colon tissue with KCl–HCl, E_1 and E_2 = same as in Fig. 1, S_1 = immune serum against E_1, and S_2 = immune serum against E_2.

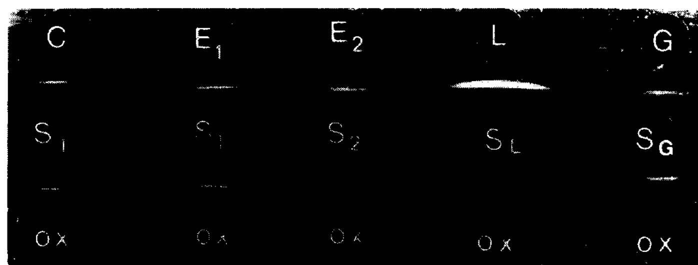

Fig. 3. Agar diffusion plate: L = lactosyl bovine serum albumin, SL = anti-lactose serum, G = glucose oxidase, S_G = anti-glucose oxidase serum, Ox = periodate oxidized antigens of the preparations in the top wells and remaining abbreviations as in Figs. 1 and 2.

protein band and no other protein-staining compounds. Likewise the diffusion pattern showed only one precipitin arc and no additional precipitin arcs are shown. Evidently proteolysis of CEA did not occur and the new protein-staining components in the tumor extract (E_1, Fig. 1) are most likely native components of the tumor tissue. These components also yielded precipitin arcs in agar diffusion tests against immune serum (Fig. 1). Comparison of the molecular sizes of the various antigens was not made due to the limited amounts available. Although a comparison of the coupled method and the Western Blot method used for detecting tumor antigens was not made, the coupled method is probably more selective and sensitive because it relies on the magnitude of the charge, the charge diffusion rate and the rate of complex formation. The Western Blot method relies on complex formation only. The coupled method has been used successfully for the analysis of isomeric antibodies produced by immunization of rabbits with a glycoconjugate of fucose and bovine serum albumin [30]. The isomeric antibodies were separated on polyacrylamide gels by isoelectric focusing. In view of the above, the coupled method can likely be used for analyzing dissociative gels.

The presence of several new tumor antigens in human colon with adenocarcinoma is demonstrated by the electrophoresis–agar diffusion coupled method. Such antigens are absent in extracts of normal colon tissue. The method is readily adaptable to analysis of tumors of other organs [31].

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