Carcinoembryonic Antigen-Like Substances of Human Urothelial Carcinomas

ISOLATION OF COMPONENTS FROM PATHOLOGICAL URINE AND COMPARISON WITH COLORECTAL CARCINOMA ANTIGENS

R. NERY, A. L. BARSOUM*, H. BULLMAN and A. M. NEVILLE
*Division of Pathology, Institute of Cancer Research: Royal Cancer Hospital,
Chester Beatty Research Institute, Fulham Road, London SW3 6JB, U.K.*

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Urines of several patients with urothelial carcinomas contain inhibitors of the immuno-reaction between carcinoembryonic antigen derived from human colorectal carcinomas and monospecific goat antiserum raised against the antigen. These inhibitors range in approximate molecular weights from less than 1000 to several millions, and two have been isolated by a combination of extraction, gel filtration and electrophoretic procedures. These are respectively a macromolecular aggregate, component UCEA-3, which is excluded by Sepharose 4B, and a glycoprotein(s) component, UCEA-1, with mean molecular weight (2×10^5) similar to that of carcinoembryonic antigen. Comparison of the properties of component UCEA-1 and carcinoembryonic antigen on gel filtration, electrophoresis, immunoelectrophoresis and density gradient ultracentrifugation indicates that these substances of similar molecular size and net charge differ in some immunochemical properties.

Carcinoembryonic antigen, a glycoprotein first described by Gold & Freedman (1965*a,b*) was originally thought to be a tumour-specific antigen found only in carcinomas and normal foetal tissues of endodermal origin. Improved assay techniques (Thomson *et al.*, 1969; LoGerfo *et al.*, 1971; Egan *et al.*, 1972) showed that the concentration of the circulating antigen may decrease significantly after tumour resection and increase on tumour recurrence (LoGerfo *et al.*, 1972; Laurence *et al.*, 1972). However, such materials may be found in the serum, faeces, urine and meconium of normal healthy subjects and in the serum of patients with a wide variety of non-neoplastic regenerative or inflammatory disorders and with non-endodermally derived benign and malignant neoplasms (Martin & Martin, 1970; Moore *et al.*, 1971, 1972; Freed & Taylor, 1972; Hall *et al.*, 1972; Laurence *et al.*, 1972; Reynoso *et al.*, 1972; Kupchik & Zamcheck, 1973; Neville *et al.*, 1973).

The wide distribution of carcinoembryonic antigenic activity was ascertained primarily by radio-immunoassay, which measures only the degree of inhibition of the immunoreaction between carcinoembryonic antigen derived from colorectal carcinoma and its immunospecific antiserum. It is thus possible that some cross-reacting antigenic determinants are

present that may be capable of independent immunological determination. In this connection, it has been shown (Holburn *et al.*, 1973) that carcinoembryonic antigen of endodermally-derived carcinomas may contain incomplete antigenic determinants of the ABO blood-group system. The present paper describes some substances that occur in the urine of patients with urothelial (bladder) carcinoma, and which are immunologically cross-reactive with the carcinoembryonic antigen.

Materials

Chemicals and reagents

Sepharose and Sephadex gels and Blue Dextran 2000 were obtained from Pharmacia (G.B.) Ltd., London W.5, U.K.; gelatin-treated cellulose diacetate strips and blocks (CelloGel) from Reeve Angel Scientific Ltd., London S.E.1, U.K.; protein standards (Collection MS-11) and agarose were from Serva, Heidelberg, West Germany; acrylamide and *NN'*-methylenebisacrylamide were from BDH Chemicals Ltd., Poole, Dorset, U.K.; and rabbit immunoglobulin G was from Wellcome Research Laboratories, Beckenham, Kent, U.K. Other chemicals and reagents were obtained from various commercial sources.

Apparatus

Chromatographic columns equipped with cooling jackets were obtained from Pharmacia (G.B.) Ltd.

* Present address: Department of Medical Virology and Immunology, University of Essen, Hufelandstrasse, Essen, West Germany.

The electrophoresis apparatus (model U77) and analytical polyacrylamide-gel-electrophoresis apparatus were from Shandon Scientific Co. Ltd., London N.W.10, U.K., and ultrafiltration apparatus (model 2000) and membranes were from Amicon Ltd., High Wycombe, Bucks., U.K.

Aqueous buffer solutions

Phosphate-buffered saline solutions contained NaH_2PO_4 (50mmol), NaCl (150mmol) and NaN_3 (3.1mmol) per litre of solution adjusted to pH 5.8 or 7.2 with 1M-NaOH. Phosphate-buffered saline-EDTA-rabbit immunoglobulin G (pH 7.2) contained NaH_2PO_4 (22.5mmol), Na_2HPO_4 (52.5mmol), NaCl (75mmol), EDTA (0.8mmol) and rabbit immunoglobulin G (1g) per litre of solution. Veronal buffer (pH 8.6) contained sodium veronal (50mmol) and veronal (10mmol) per litre of solution. Borate buffer (pH 8.6) contained $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (25.5mmol) and boric acid (75mmol) per litre of solution. Borate-buffered urea (pH 8.6) contained urea (5mol) per litre of borate buffer.

Urines

These were obtained from patients with urothelial carcinoma at the Royal Marsden Hospital. The specimens were collected in sterile containers and stored at -20°C until required.

Goat antiserum

This, monospecific for carcinoembryonic antigen derived from colorectal carcinoma, was prepared from goats immunized with a standard purified preparation of the antigen as described by Darcy *et al.* (1973).

Carcinoembryonic antigen

All preparations were from human colorectal carcinomas or their liver metastases. The standard preparation was a gift from Dr. C. W. Todd (City of Hope Medical Center, Duarte, Calif., U.S.A.), and was labelled with ^{125}I as described by Egan *et al.* (1972). Other samples, kindly supplied by Dr. C. Turberville (Chester Beatty Research Institute) were prepared as described by Krupey *et al.* (1968) (Scheme 1). The antigen is a glycoprotein, or a mixture of glycoproteins, of average mol.wt. 2×10^5 . Briefly, the antigen was prepared by extraction of homogenized tumour tissue with aqueous 1M- HClO_4 and fractionation of the solubilized components by successive gel filtration on columns (10cm $\times$ 89cm) of Sepharose 4B and Sephadex G-200 (Scheme 1). Unless otherwise indicated, carcinoembryonic antigen refers to preparations obtained

in this way, without further purification. Generally such materials show 60–100% of the activity in radioimmunoassay of the more highly purified standard substance (Scheme 1c).

Methods

Radioimmunoassay

This was performed by the double-antibody procedure of Egan *et al.* (1972) as modified by Laurence *et al.* (1972). Briefly, inhibitor in phosphate-buffered saline-EDTA-rabbit immunoglobulin G (0.2ml) was mixed with suitably diluted monospecific goat anti-(carcinoembryonic antigen) antiserum (0.05ml) and a solution of ^{125}I -labelled standard antigen (1–2ng; 3×10^4 c.p.m.) in the same buffer (0.01ml) containing kanamycin (26 μg). The radioactivity of the mixture was determined with a gamma counter. The mixture was incubated overnight at 37°C , mixed with undiluted horse anti-(goat immunoglobulin G) antiserum (0.1ml), incubated for 1h at 37°C , kept at 4°C for 15min and centrifuged. The supernatant was discarded and the radioactivity of the sediment determined as described above. The ratio of the radioactivities obtained before and after centrifugation gave the fraction of bound radioactivity; this, expressed as a fraction of the binding observed when a control solution containing buffer (0.2ml) in place of inhibitor was treated similarly, gave the percentage inhibition of binding. The antigenic activity of the inhibitor was calculated by reference to a standard curve in which known quantities of determined concentration-dependent activity of unlabelled standard antigen were added to incubation mixtures containing iodinated standard antigen and goat antiserum.

The carcinoembryonic antigen-like activity of urinary preparations is expressed as weight-equivalent antigenic activity, this being arbitrarily defined as that weight or concentration of the standard antigen required to give an equivalent inhibition in the radioimmunoassay. It is thus a measure to which the urinary sample competes with the standard antigen for the specific binding sites of the monospecific anti-(carcinoembryonic antigen) antibodies. This competition may be effected by shared structurally similar, or by structurally dissimilar, cross-reacting antigenic determinants.

Staining

Cellulose diacetate (Cellogel) or filter paper strips or polyacrylamide gels were stained with Nigrosine or Coomassie Brilliant Blue until the bands were revealed (usually overnight). Carbohydrate components were revealed on Cellogel and paper strips or in polyacrylamide gels by the periodate-Schiff

Scheme 1. Flow diagram of the fractionation of carcinoembryonic antigen-like components of the urine of a patient with bladder carcinoma

Each set of four values (where shown) indicates per litre of urinary supernatant: (i) weight (mg) of freeze-dried product, (ii) weight (µg) and (iii) percentage (w/w) of antigenic activity of the product and (iv) percentage (w/w) recovery of antigenic activity in the purification step, in the order given. For comparison, the percentage (w/w) of antigenic activity of HClO₄ extracts of several human colorectal carcinomas and their metastases (Krupey *et al.*, 1968; Coligan *et al.*, 1972; Turberville *et al.*, 1973) at corresponding stages of purification is also included. Antigenic activities were determined by the modified (Laurence *et al.*, 1972) double-antibody radioimmunoassay procedure of Egan *et al.* (1972). In this, incubation of the test substance and ¹²⁵I-labelled carcinoembryonic antigen from colorectal carcinomas with goat monospecific anti-(carcinoembryonic antigen) antiserum is followed by precipitation of the immunoglobulin G antibodies and their immune complexes with horse anti-(goat immunoglobulin G) antiserum; the amount of bound radioactivity in the precipitate, by reference to suitable controls and standards, gives the antigenic activity of the sample. (*a*) Fractionation by Cellogel-block electrophoresis in borate buffer for 2h, (*b*) fractionation by centrifugation on a CsCl density gradient, (*c*) fractionation by DEAE-cellulose chromatography. For other details see the text.

procedures of Bodman (1968) or Zacharius *et al.* (1969), respectively.

Ultrafiltration

Urine samples were clarified by centrifugation at 2200g at 4°C for 30min and the supernatant solutions were filtered in the Amicon ultrafiltration apparatus with stirring at 4°C.

Gel-filtration chromatography

The packed columns were developed overnight or longer with phosphate-buffered saline (pH 5.8) at 4°C until a reproducible void volume (V_0), as determined by the exclusion of Blue Dextran 2000, and linear plots of K_{av} , (i.e. $V_e - V_0 / V_t - V_0$) versus

log(molecular weight) of suitable protein standards were obtained: V_e and V_t were the elution volume and total volume respectively of the column. Fractions (25ml) were collected from a Sepharose 4B column (bed dimensions 10cm×80cm; V_0 1.625 litres) and fractions (8.5ml) from a Sephadex G-200 column (bed dimensions 7.5cm×84cm; V_0 470ml) at a flow rate of 1.3-1.5ml/h per cm² cross-sectional area at 4°C. The E_{280} of effluent fractions was continuously recorded with an LKB Uvicord recorder or manually with a Unicam SP.800 u.v. spectrophotometer. The antigenic activity of selected fractions was determined as a routine by radioimmunoassay. Appropriate fractions were combined, dialysed at 4°C for 3 days against several changes of deionized double-distilled water and freeze-dried.

Electrophoresis on Cellogel

Cellogel blocks (6cm×17cm×2.5mm) or strips (2.7cm×14cm or 5.7cm×11cm) were soaked overnight in buffer and blotted between sheets of Whatman 3MM filter paper. The sample was evenly distributed on a Cellogel block with a Hamilton microlitre syringe, with its tip cut off and blunted, at a distance (2cm) from the cathodic end of the block along pinholes (0.4mm deep and 2mm apart). The ends of the block were connected to the buffer compartments of the electrophoresis apparatus with bridges of Whatman 3MM filter paper. Electrophoresis was performed at 20°C at 24mA for 2h or 4h in veronal or borate buffer, pH8.6. The block was cut into transverse sections 5mm wide, except for the first section, which extended from -0.5cm to +0.5cm towards the anode. Each section was placed in a numbered test tube, the absorbed liquid was squeezed out with a glass rod with a thickened end and the antigenic activity of the exudate was determined by radioimmunoassay. Fractions containing antigenic activity were combined. The Cellogel slices were loosely packed into a small chromatographic column (1cm×20cm) and eluted with water (about 30ml) until no further activity was eluted. The eluate was filtered through Whatman no. 42 filter paper and de-salted by passage through a small column (1cm×20cm) of Sephadex G-25. The Sephadex G-25 column was further eluted with water (15ml) and the effluents were combined and dialysed in Visking tubing against several changes of deionized double-distilled water for 2 days at 4°C and freeze-dried. Protein and glycoprotein bands on electrophoretograms were located by pressing duplicate 1cm-wide strips of Whatman no. 1 filter paper along the length of the block followed by staining of the resulting 'prints' with Coomassie Brilliant Blue and the periodate-Schiff reagent respectively. For electrophoresis on Cellogel strips, the samples were applied as discrete spots approx. 4cm from the cathodic end of the strips with the blunt Hamilton microlitre syringe. The strips were connected to the buffer compartments with Whatman 3MM filter-paper bridges. For immunoelectrophoresis on Cellogel strips, the samples were applied similarly, 2cm apart; after electrophoresis, strips of Whatman no. 1 filter paper (2mm wide) evenly wetted with the undiluted antiserum (40–100μl) were placed lengthwise on the electrophoretogram at a distance (9mm) from the edge of the strip to the line of development of the sample. The Cellogel strip was placed over a hole cut slightly smaller than the dimensions of the strip through a wad of Whatman 3MM filter paper wetted with the electrophoresis buffer and placed in a moist chamber for 60–72h at room temperature. The immunoelectrophoretogram was washed by agitation in several changes of phosphate-buffered saline

(pH7.2) during 8–16h and stained with Nigrosine. Unlike immunodiffusion in agarose (see below), precipitin lines on Cellogel were visible only after staining.

Disc electrophoresis on polyacrylamide gels

This was done by the method of Ornstein & Davis (Davis, 1964). The sample (20–30μg) was dissolved in the appropriate electrophoresis buffer (10–15μl) containing, where appropriate, dissolved urea (50–75μmol) and/or dithiothreitol (0.4–0.6μmol).

Immunodiffusion in agarose

This was done on glass microscope slides (7.6cm×2.5cm) coated to a depth of 2mm with a 1.5% (w/v) solution of agarose in veronal buffer. Preliminary immunotitration experiments were performed to establish optimum dilutions of antigen and antiserum required for the formation of visible immunoprecipitates.

CsCl density-gradient centrifugation

This was done in an MSE Superspeed 65 centrifuge by using a 3×5ml aluminium swing-out rotor with a maximum relative centrifugal force of 178000g. Samples were dissolved in 0.1M-sodium phosphate buffer (pH7.4; 5ml) containing CsCl (2.9g) and centrifuged at 120000g at 4°C for 80h. The density at 25° (ρ^{25}) of each fraction (16 drops) collected from the bottom of the centrifuge tube was calculated from the refractive index at 25°C (n_D^{25}) by using the relationship: $\rho^{25} = 10.8601n_D^{25} - 13.4974$ (Ifft *et al.*, 1961).

Results

Fractionation of urinary components with carcino-embryonic antigen-like activity

Ultrafiltration. A sample (100ml) of the supernatant solution obtained after centrifugation of urine from a patient with bladder carcinoma was ultrafiltered through a UM-2 filter. The residue was dissolved in phosphate-buffered saline (pH7.2; 50ml) and the solution ultrafiltered successively through XM-300, XM-100A and PM-30 filters. The washed residue on each filter was dissolved in phosphate-buffered saline (pH7.2; 100ml). The antigenic activity as determined by radioimmunoassay was distributed in all fractions (Table 1) with the largest proportion (83%) having an approximate mol.wt. greater than 3×10^5 . A sample (90ml) of the fraction retained by the XM-300 filter, i.e. of mol.wt. above 3×10^5 , was treated at 4°C for 30min with an equal volume of 2M-HClO₄ and the soluble fraction was isolated by

Table 1. Fractionation by ultrafiltration of carcinoembryonic antigen-like components of urine from a patient with bladder carcinoma

(A) The supernatant solution (100ml), obtained after centrifugation of urine at 2200g for 30min, was ultrafiltered through the UM-2 filter. The eluate (fraction 1) was kept and the residue was dissolved in phosphate-buffered saline (pH 7.2, 50ml) and ultrafiltered successively through XM-300, XM-100A and PM-30 filters. The residue on each filter (respectively fractions 2-5) was dissolved in the same buffer (100ml). (B) A HClO_4 -soluble fraction (HClO_4 extract) of the solution of the material retained by the XM-300 filter (i.e. of the fraction having an approx. mol.wt. over 3×10^5) was ultrafiltered successively through the last three filters as for (A) and each residue was dissolved in phosphate-buffered saline (pH 7.2; 25ml). (C) The residue (3.5g wet wt.), obtained after centrifugation of the same urine (10 litres), was suspended in glycine-HCl buffer (0.1M, pH 2.0, 35ml) and incubated at 37°C for 30min. The incubation mixture was centrifuged at 25000g at 4°C for 1h and the supernatant (glycine buffer extract) was dialysed against water, ultrafiltered successively through the last three filters as above, and each residue was dissolved in phosphate-buffered saline (pH 7.2; 25ml). (D) An aqueous solution (20ml) of carcinoembryonic antigen (10µg) purified from colorectal carcinoma on Sephadex G-200 (CEA solution) was ultrafiltered successively through XM-300, XM-100A, PM-30 and UM-2 filters and each residue was dissolved in phosphate-buffered saline (pH 7.2, 50ml). The antigenic activity of the various subfractions was determined by radioimmunoassay.

Fraction no.	Approximate molecular weight	Distribution of antigenic activity (% of total)			
		A (urinary supernatant)	B (HClO_4 extract)	C (glycine buffer extract)	D (CEA solution)
1	$<10^3$	4	—	—	0
2	$10^3-3 \times 10^4$	8	9.5*	5*	1
3	$3 \times 10^4-10^5$	4	6.5	2	1
4	$10^5-3 \times 10^5$	1	59	74.5	99
5	$>3 \times 10^5$	83	25	18	0.2

* Activity of fraction of approx. mol. wt. under 3×10^4 .

centrifugation. The dialysed and freeze-dried soluble fraction, containing 81 µg wt. equivalents of antigenic activity, was ultrafiltered successively through XM-300, XM-100A and PM-30 filters. The residue on each filter was dissolved in phosphate-buffered saline (pH 7.2; 25ml) and the antigenic activity of each fraction was determined by radioimmunoassay. This again showed (Table 1) a distribution of activity in all the fractions with 59% now appearing in the approximate mol.wt. range $10^5-3 \times 10^5$. This implies that the macromolecular species previously retained by the XM-300 filter was either acid-labile or an aggregate containing some acid-dissociable carcinoembryonic antigen-like components of various smaller molecular sizes, but mainly of molecular weight between 10^5 and 3×10^5 .

To determine whether some of the antigenic activity was also bound in an insoluble acid-dissociable form, for instance to cellular components or as immune complexes, urine (10 litres) from a patient with bladder carcinoma was centrifuged at 2200g at 4°C for 30min, the residue was resuspended in water (50ml) and the suspension was recentrifuged as described above. This washing procedure was repeated until the final wash contained no antigenic activity as determined by radioimmunoassay. The washed residue (3.5g wet wt.) was suspended in glycine-HCl buffer (0.1M, pH 2.0, 35ml) and incubated with shaking at 37°C for 30min. The

incubation mixture was centrifuged at 25000g at 4°C for 1h and the supernatant dialysed in Visking tubing for 2 days against five changes of water (500ml each). Ultrafiltration of the solution in the tubing, containing 2.94 µg weight equivalents of antigenic activity, through the XM-300, XM-100A and PM-30 as described above showed (Table 1) that almost all of the activity appeared within the approximate mol.wt. range $10^5-3 \times 10^5$.

Gel filtration on Sepharose 4B. A 5-litre Buchner flask was fitted with a glass funnel and the outlet of the flask was connected to a water pump. One end of a length (approx. 1.5m) of Visking tubing, contained in the flask, was connected to the stem of the funnel and the other was knotted. At 4°C, the urine was gradually introduced through the funnel into the tubing while the flask was gently evacuated at the water pump. The concentrated fraction (approx. 2.5 litres) in the tubing from urine (24 litres) from a patient with bladder carcinoma was mixed at 4°C with an equal volume of 2M- HClO_4 and centrifuged after a further 30min. The soluble fraction was dialysed and freeze-dried. A sample (3.6g) of the freeze-dried product (4.85g) was dissolved in phosphate-buffered saline (pH 5.8; 70ml) and chromatographed on a column (10cm × 80cm) of Sepharose 4B by elution with the same buffer (Fig. 1). Radioimmunoassay of effluent fractions from V_e/V_0 0.8 to 3.6 showed two main antigenically active peaks;

Fig. 1. Sepharose 4B chromatography of a HClO_4 -soluble fraction of urine from a patient with bladder carcinoma

The urine (24 litres) was concentrated under reduced pressure at 4°C and a HClO_4 -soluble fraction of the concentrate was prepared. A sample (3.6g) of the dialysed and freeze-dried acid-soluble fraction (4.85g) was fractionated on a column ($10\text{cm} \times 80\text{cm}$) of Sepharose 4B by elution with phosphate-buffered saline (pH 5.8; 0.05M). The antigenic activity (○), arbitrarily defined as that weight or concentration of a standard preparation of carcinoembryonic antigen derived from colorectal carcinoma required to give an equivalent activity in the radioimmunoassay, and the E_{280} (—) of effluent fractions (25ml) were determined. The elution (●) of antigenic activity from a sample (5mg) of carcinoembryonic antigen from colorectal carcinoma is also shown.

one, designated component UCEA-3, appeared in the void volume and the other, designated component UCEA-1, at V_e/V_0 2.40. Carcinoembryonic antigen derived from colorectal carcinoma was eluted from the same column, under the conditions described, at V_e/V_0 2.36. Freeze-dried products (1.75g and 0.126g) were obtained from the peaks containing components UCEA-1 (V_e/V_0 2.2–2.6) and UCEA-3 (V_e/V_0 0.9–1.2) respectively. Radioimmunoassay indicated that these contained 0.5% (w/w) and 0.3% (w/w) of the activity of the standard carcinoembryonic antigen, representing 60% and 2.6% respectively of the total activity placed on the column (Scheme 1).

In another experiment, the urine concentrate was dialysed in Visking tubing against several changes of water at 4°C for 3 days. The non-diffusible fraction was freeze-dried and the residue (3.2g) fractionated through the same Sepharose 4B column under the conditions described above. Radioimmunoassay of effluent fractions showed two main peaks of antigenic activity centred at V_e/V_0 1.1 and V_e/V_0 2.4. The effluents within the V_e/V_0 range 0.9–4.2 were combined into four fractions with cut-off points at the

following V_e/V_0 values: 1.2, 2.3, 2.7. Radioimmunoassay showed that the resulting four fractions contained approx. 8, 17, 60 and 15% of the total antigenic activity eluted from the column.

Gel filtration on Sephadex G-200. The freeze-dried product (1.75g) containing fraction UCEA-1 obtained from Sepharose 4B chromatography (Fig. 1) was dissolved in phosphate-buffered saline (pH 5.8; 10ml) and chromatographed on a column ($7.5\text{cm} \times 84\text{cm}$) of Sephadex G-200. A broad peak of antigenic activity, extending from V_e/V_0 1.0 to 1.6, was obtained; this gave a freeze-dried product (324mg) having an antigenic activity of 2.2% (w/w). Rechromatography of a sample (198mg) of this product, as described above, gave a major peak of antigenic activity (Scheme 1) centred at V_e/V_0 1.20. The combined fractions (V_e/V_0 1.1–1.25) gave a freeze-dried product (165mg) with an antigenic activity of 2.5% (w/w). Unless otherwise stated, further experiments with fraction UCEA-1 refer to this preparation. A solution of carcinoembryonic antigen (2mg) derived from colorectal carcinoma in phosphate-buffered saline (pH 5.8; 5ml), after

similar chromatography, showed a peak of antigenic activity centred at V_e/V_0 1.18.

Cellogel block electrophoresis. Fraction UCEA-1 (7.5mg) in borate buffer (pH8.6; 0.4ml) was subjected to electrophoresis on Cellogel blocks at 24mA for 35min. A similar sample was electrophoresed for 2h in the same buffer. Fraction UCEA-1 (7.5mg) in veronal buffer (pH8.6; 0.4ml) was similarly treated for 4h in veronal buffer. The results showed a major glycoprotein band coincident with the peak of antigenic activity at 0.5–2.0, 2.0–3.5 and –0.5–2.0cm from the origin towards the anode for the three samples in the order given. These gave freeze-dried products (4.2mg, 2.9mg and 5.0mg) with 2.8, 3.0 and 2.5% (w/w) respectively of the antigenic activity of the standard carcinoembryonic antigen.

Immunodiffusion in agarose

Fraction UCEA-1 (150 μ g) obtained from Sephadex G-200 chromatography of urine from a female patient with bladder carcinoma, or carcinoembryonic antigen (15 μ g) derived from colorectal carcinoma in veronal buffer, pH8.6 (10 μ l), after immunodiffusion in a moist chamber at room temperature for 60h against monospecific goat anti-(carcinoembryonic antigen) antiserum (30 μ l, undiluted) showed precipitin lines of antigenic identity. However, variations in this antigenic property of carcinoembryonic antigen-like substances in the urines of individual patients with bladder carcinoma may occur; in one case, lines of partial antigenic identity were seen (Dr. D. Darcy, personal communication) and, in at least three others, precipitin lines of apparent antigenic identity were produced with difficulty. The lower limit of detection of the antigen(s) by this method is at least 1000 times greater than that achieved by radioimmunoassay. This low sensitivity, which is further decreased by the low (i.e. 3%) specific (w/w) antigenic activity of the urinary preparations, may account for the difficulty.

Electrophoresis and immunoelectrophoresis on Cellogel strips

Fraction UCEA-1 (200 μ g) or carcinoembryonic antigen (15 μ g) derived from colorectal carcinoma was dissolved in borate buffer (4 μ l). The samples were subjected to electrophoresis in the same buffer at 2mA/cm strip width for 18min. One electrophoretogram was stained for glycoprotein with the periodate–Schiff reagent, a second for protein with Coomassie Brilliant Blue and a third was subjected to immunodiffusion against monospecific goat anti-(carcinoembryonic antigen) antiserum and stained with Nigrosine. Both samples showed a major coincident protein, glycoprotein and antigenically active band with a mean electrophoretic mobility

of 52mm/h per mA. When veronal buffer replaced the borate buffer, fraction UCEA-1 and carcinoembryonic antigen migrated as diffuse bands having a mean electrophoretic mobility of 5.3mm/h per mA.

Electrophoresis in polyacrylamide gels

Preliminary experiments with fraction UCEA-1 and using separation gels of 5, 7.5, 10, 15, 25 and 30% (w/v) were performed in veronal or borate buffer, in either the presence or the absence of urea or of dithiothreitol. The results indicated that the sharpness of the periodate–Schiff-stained bands decreased and their mean migration distances increased with decreasing gel concentration. The glycoprotein migrated more slowly in the veronal than in the borate buffer, other conditions being equal. Treatment of fraction UCEA-1 or carcinoembryonic antigen with dithiothreitol before electrophoresis in

Fig. 2. Electrophoresis on polyacrylamide gels of component UCEA-1 and carcinoembryonic antigen derived from colorectal carcinoma

Component UCEA-1, obtained as a Sepharose 4B fraction (Fig. 1), was chromatographed twice on a column (7.5cm $\times$ 84cm) of Sephadex G-200. A sample (20 μ g) of the dialysed and freeze-dried final product or of the carcinoembryonic antigen obtained as a Sephadex G-200 fraction by the method of Krupey *et al.* (1968) was dissolved in borate-buffered urea (pH8.6; 10 μ l) and subjected to electrophoresis in the same buffer in 15% (w/v) polyacrylamide gels at 3mA/gel for 7h. The gels were stained for glycoproteins by the periodate–Schiff procedure of Zacharius *et al.* (1969). (1) Carcinoembryonic antigen; (2) component UCEA-1.

borate buffer or in borate-buffered urea at 3 mA/gel for 7 h did not cause a change in the electrophoretic mobility of either substance. General protein stains such as Coomassie Brilliant Blue, Nigrosine and Amido Black were less sensitive for the glycoproteins than was the periodate-Schiff stain. A sample (20 μ g) of fraction UCEA-1 or of carcinoembryonic antigen dissolved in borate-buffered urea (10 μ l) was

subjected to electrophoresis in this buffer in 15% (w/v) gels at 3 mA/gel for 7 h and the gels were stained by using the periodate-Schiff procedure of Zacharius *et al.* (1969). Apparently identical bands (Fig. 2) were revealed for both substances with electrophoretic mobilities of 0.67 mm/h per mA.

Heterogeneity of fraction UCEA-1 and of carcinoembryonic antigen derived from colorectal carcinoma

Centrifugation on a CsCl density gradient of a sample of component UCEA-1 (50 μ g) or of carcinoembryonic antigen (5 μ g) showed three peaks of antigenic activity at densities of 1.58, 1.54 and 1.48 for component UCEA-1 and 1.58, 1.53 and 1.48 for the other antigen in increasing order of relative proportions. A sample (2 μ g) of carcinoembryonic antigen obtained from the apex of a DEAE-cellulose chromatographic peak (Turberville *et al.*, 1973), and a sample (20 μ g) of fraction UCEA-1, further purified by electrophoresis on a Cellogel block in borate buffer for 2 h, showed a relative loss of the less dense components of both substances (Fig. 3).

Discussion

Human colorectal adenocarcinomas or their metastases are known to contain at least three glycoproteins giving positive values in radioimmunoassay for the carcinoembryonic antigen derived from colorectal carcinomas. These include that described initially by Gold & Freedman (1965*a,b*), a second of lower molecular weight (Mach & Pusztaszeri, 1972; von Kleist *et al.*, 1972; Darcy *et al.*, 1973; Turberville *et al.*, 1973) and a third, isolated from a liver metastasis of a human colonic carcinoma of larger molecular size (Coligan *et al.*, 1972). The second glycoprotein also occurs in other tumours and in the urines of patients with urothelial carcinomas (Darcy *et al.*, 1973). The present report indicates that such urines also contain, among others, a carcinoembryonic antigen-like glycoprotein of size similar to that of the first antigen and a macromolecular aggregate which appears to be different from the third.

The urines of patients with urothelial carcinoma may contain abnormally increased amounts of substances giving positive values in radioimmunoassay for the carcinoembryonic antigen derived from colorectal carcinoma; these amounts may be further increased if urinary infection or contamination of the urines with vaginal or cervical secretions from these patients is present (Hall *et al.*, 1972). The present results show that the urinary materials measured by radioimmunoassay (Hall *et al.*, 1972) consist of several species ranging in apparent molecular weights from less than 10^3 to over 2×10^7 . Moreover, fractionation of infected and non-infected urines from some 40 patients with urothelial carcinoma by gel

Fig. 3. Density-gradient ultracentrifugation of component UCEA-1 and carcinoembryonic antigen derived from colorectal carcinoma

The following samples were analysed: (a) component UCEA-1 (50 μ g), obtained after dialysis against water and freeze-drying of the rechromatographed Sephadex G-200 fractions, $\blacktriangle$; (b) component UCEA-1 (20 μ g) obtained after further purification of sample (a) by Cellogel-block electrophoresis, $\bullet$; (c) carcinoembryonic antigen derived from colorectal carcinoma (5 μ g) obtained after Sephadex G-200 chromatography as described by Krupey *et al.* (1968), $\triangle$; (d) carcinoembryonic antigen (5 μ g) obtained after further purification of sample (c) by DEAE-cellulose chromatography as described by Turberville *et al.* (1973), $\square$; Each sample was dissolved in 0.1 M-sodium phosphate buffer (pH 7.4, 5 ml) containing CsCl (2.9 g) and centrifuged at 95000*g* for 80 h. The density ($\circ$) at 25°C of consecutive fractions (16 drops) collected from the bottom of the centrifuge tube was determined from the refractive index as described by Ifft *et al.* (1961), and the antigenic activity of the fractions was determined by radioimmunoassay. Comparison of the curves given by samples (a) and (b) shows a relative loss, in sample (b) of the component sedimenting at density 1.48 as a result of the further purification step. A similar loss of this component is seen in sample (d) after further purification of sample (c).

filtration on Sepharose 4B or by Amicon ultrafiltration showed that the sum of the radioimmunoassay titres of the fractions from a particular urine was significantly different from the radioimmunoassay titre of the same unfractionated urine (R. Nery, A. L. Barsoum & H. Bullman, unpublished work). These results may be relevant to the unsatisfactory correlations observed (Hall *et al.*, 1972) between the radioimmunoassay titre of the urine and the clinical diagnosis of the patient.

Aggregates containing carcinoembryonic antigen-like components are apparently excreted in the urines of patients with urothelial carcinoma. Fraction UCEA-3, obtained in the void volume during Sepharose 4B chromatography and therefore having an approximate molecular weight of over 2×10^7 , contains carcinoembryonic antigen-like activity (Fig. 1) and immunoglobulins G, A and M (R. Nery, A. L. Barsoum & H. Bullman, unpublished work). Ultrafiltration of urine from a patient with bladder carcinoma showed that the bulk (83%) of the carcinoembryonic antigen-like activity as determined by radioimmunoassay was recovered in the fraction of approximate mol.wt. over 3×10^5 (Table 1, fraction 5). A HClO_4 -soluble extract of this fraction, when refractionated by ultrafiltration, showed that approx. 59% of the radioimmunoactivity appeared within the apparent molecular-weight range 10^5 – 3×10^5 . This indicated that one effect of the extraction was to release some antigenic activity from a larger aggregate. Carcinoembryonic antigen-like substances may also be solubilized from insoluble components of such urines, by treatment at 37°C with aqueous glycine buffer, pH 2.0; most (74.5%) of the solubilized antigenic activity appeared within the apparent molecular-weight range 10^5 – 3×10^5 (Table 1).

Gel filtration on Sepharose 4B of a HClO_4 -soluble fraction of the urine from a patient with bladder carcinoma showed two main antigenically active components: fraction UCEA-3, already discussed, and fraction UCEA-1. The latter occurred in greater proportion, was eluted almost coincidentally with carcinoembryonic antigen derived from colorectal carcinoma and had an approximate mol.wt. of 2×10^5 (Fig. 1). Considerable purification of fraction UCEA-1 was achieved by repeated gel filtration on Sephadex G-200 followed by electrophoresis on Cellogel blocks. Radioimmunoassay showed that the final product contained 3% (w/w) of the antigenic activity of an equal weight of standard carcinoembryonic antigen. In contrast, preparations of carcinoembryonic antigen from colorectal carcinomas contained 40–160 times the antigenic activity (w/w) of preparations of component UCEA-1 at parallel stages in the purification procedures. The results are summarized in Scheme 1.

In a number of properties, fraction UCEA-1 obtained from Sephadex G-200 was qualitatively

similar to, but quantitatively different from, carcinoembryonic antigen obtained from colorectal carcinoma. These differences and similarities were detected by electrophoresis and immunoelectrophoresis on Cellogel, electrophoresis in polyacrylamide gels (Fig. 2), immunodiffusion in agarose, radioimmunoassay (Scheme 1) and density-gradient ultracentrifugation (Fig. 3). The heterogeneity of the two preparations, as revealed by ultracentrifugation, is similar to that described (von Kleist & Burtin, 1969; Krupey *et al.*, 1968; Turner *et al.*, 1970) for various preparations of carcinoembryonic antigen derived from colorectal carcinoma; further purification of the Sephadex G-200 fraction of component UCEA-1 by Cellogel-block electrophoresis or of the corresponding fraction of carcinoembryonic antigen of colorectal carcinoma by DEAE-cellulose chromatography resulted in a partial loss of the lighter component in each case (Fig. 3 and Scheme 1). Heterogeneity of carcinoembryonic antigen derived from colorectal carcinomas has also been observed (Coligan *et al.*, 1973) during isoelectric focusing and ECTEOLA-cellulose chromatography of Sephadex G-200 fractions of the antigen. Similar fractions of component UCEA-1 also show heterogeneity during isoelectric focusing and DEAE-cellulose chromatography (R. Nery & R. James, unpublished work).

Biological materials giving positive values in radioimmunoassays for carcinoembryonic antigen derived from colorectal carcinomas may thus represent a heterogeneous population of antigenically related substances. They occur in glycolipid fractions, freed from non-lipid contaminants by chromatography on Sephadex G-25, of human erythrocyte membranes (Nery *et al.*, 1973) and in glycoproteins of different structures both in the constituent heteropolysaccharide chains and in the protein backbones (Coligan *et al.*, 1973). Further, these glycoproteins may carry multiple antigenic specificities, e.g. of blood group (Holburn *et al.*, 1973), carcinoembryonic antigen-like and other (Mach & Pusztaszeri, 1972) specificities. The biological heterogeneity of carcinoembryonic antigen derived from human colorectal carcinoma has been demonstrated by metabolic studies in rabbits and dogs (Shuster *et al.*, 1973). The specificities may be properties mainly of the constituent heteropolysaccharide chains: the weight equivalent carcinoembryonic antigen-like activity of component UCEA-1 and carcinoembryonic antigen derived from colorectal carcinomas is significantly decreased after oxidation of the antigens with periodate (R. Nery & H. Bullman, unpublished work). Significant variations of this activity in different preparations of both antigens (Scheme 1) may imply corresponding differences in molecular concentrations of heteropolysaccharide chains bearing carcinoembryonic antigen-like specificity. Elucidation of the structural

relationship of these substances to one another clearly requires more detailed studies of the purified components.

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