From T-antigen to plasmalogen-derived aldehydes: The identification of a marker of colorectal cancer in human rectal mucous

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Abstract: Recently, a simple noninvasive screening test for colorectal cancer was proposed, based on a hypothesis involving galactose-containing carbohydrate moieties such as the Thomsen–Friedenreich antigen. According to the hypothesis, such carbohydrate moieties, present in the human rectal mucous of patients with colorectal cancer, can be specifically oxidized with galactose oxidase to form substances that, upon reaction with Schiff reagent, yield purple (magenta) coloured compounds. While evaluating this proposed test, we discovered that the colour formation is not due to the proposed reaction between oxidized galactose moieties present in rectal mucous and Schiff reagent. We found instead that the mucous from colorectal cancer patients contains compounds that form purple (magenta) adducts with the Schiff reagent directly, i.e., they do not require oxidation by galactose oxidase. We have identified these compounds as long-chain aliphatic aldehydes, mainly palmitic aldehyde $C_{15}H_{31}CH{=}O$ and stearic aldehyde $C_{17}H_{35}CH{=}O$. We have further found that the aldehydes originate from plasmalogens present in the phospholipid fraction of the mucous obtained from colorectal cancer patients. The aldehydes, present in plasmalogens as enol ethers, are released by the acidity of the Schiff reagent and in turn react with the Schiff reagent to form the coloured adducts. Correct identification of these markers could lead to the development of a more accurate colorectal cancer screening tool and to a deeper understanding of colorectal carcinogenesis.

Key words: T-antigen, plasmalogen-derived aldehydes, colorectal cancer marker.

Résumé : Récemment une nouvelle méthode simple et non invasive de dépistage du cancer colorectal a été proposée; elle est fondée sur une hypothèse impliquant des portions de sucre contenant du galactose, tel l'antigène de Thomsen–Friedenreich. Selon cette hypothèse, de telles portions de sucre présentes dans les muqueuses rectales de patients humains atteints d'un cancer colorectal peuvent être spécifiquement oxydées par l'oxydase du galactose pour former des substances qui réagissent avec le réactif de Schiff et qui produisent des composés colorés en violet magenta. En évaluant la méthode proposée, on a découvert que la formation de la couleur n'est pas due à la réaction suggérée du réactif de Schiff et des portions de galactose oxydé présentes dans la muqueuse rectale. On a plutôt trouvé que la muceuse de patients atteints de cancer colorectal contiennent des composés qui, avec le réactif de Schiff, forment directement des adduits colorés en violet magenta, c'est-à-dire qu'il n'est pas nécessaire de procéder par l'oxydation à l'aide d'oxydase du galactose. On a identifié ces composés comme étant des aldéhydes aliphatiques à longues chaînes carbonées, principalement l'aldéhyde palmitique, $C_{15}H_{31}CH{=}O$, et l'aldéhyde stéarique, $C_{17}H_{35}CH{=}O$. On a de plus trouvé que ces aldéhydes trouvent leur origine dans les plasmalogènes présents dans la fraction phospholipide de la muqueuse des patients atteints de cancer colorectal. Ces aldéhydes sont présents dans le plasmalogène sous la forme d'éthers énoliques, ils sont libérés par l'acidité du réactif de Schiff et ils réagissent alors avec le réactif de Schiff pour former les adduits colorés. L'identification correcte de ces marqueurs pourrait conduire au développement d'un outil de dépistage plus précis du cancer colorectal et à une meilleure compréhension du développement de ce type de cancer.

Mots clés : antigène T, aldéhydes dérivés du plasmalogène, marqueur du cancer colorectal.

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Dedicated to the memory of Professor Raymond U. Lemieux.

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Introduction

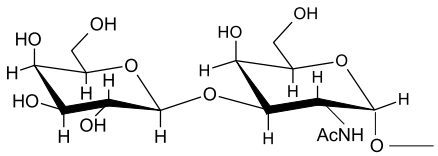
Colorectal carcinoma is the second most frequent cause of cancer mortality in men and women in industrialized countries (1, 2). Primary prevention (i.e., averting the development of the tumour by altering risk factors) is not yet feasible, since the etiology of the disease is not adequately understood. Therefore, there has recently been a great deal of interest in secondary preventive measures (i.e., detection at an asymptomatic, curable stage).

Recently, a promising screening test was proposed. It is based on the observation that most colorectal neoplasms express a disaccharide D-Galp(β1-3)-D-GalpNAcα1 (also called Thomsen–Friedenreich antigen or T-antigen) — bound as an *O*-glycoside to (-)-Ser or (-)-Thr (**I**; Scheme 1) — among the carbohydrate components of colonic mucin. This antigen is usually detected by applying fluorescently labeled peanut agglutinin (PNA) (3, 4) to histological specimens obtained from the tumour. Obviously, obtaining biopsy specimens from neoplasms inside the colon for screening purposes is not possible. Therefore, a technique (5) was proposed whereby this carbohydrate marker could be detected in colorectal mucous collected during digital rectal examination. The technique further assumes that the two galactose moieties in T-antigen are oxidizable by galactose oxidase to C-6 aldehydes in dialdehyde **II**. These two C-6 aldehydes, on subsequent reaction with Schiff reagent (6, 7), would yield an adduct detectable by its purple (magenta) colour (5, 8). Other galactose-containing oligosaccharides were subsequently added to T-antigen as carbohydrate markers of colorectal cancer (9). In addition to being simple and noninvasive, this technique has the advantage that it samples mucous from the whole colorectum because mucous from the total length of the colon flows towards the rectum. (5)

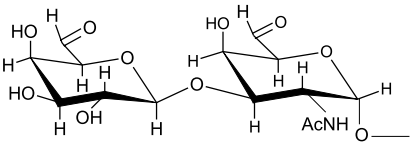
Because of the potential advantage of such a screening test, we initiated its clinical and laboratory evaluation in 1989. In 1991 we discovered unexpectedly that colorectal mucous from individuals with colorectal neoplasia developed a purple (magenta) colour after *direct* addition of Schiff reagent to the mucous specimens, without the treatment with galactose oxidase (10). The oxidation step was somewhat puzzling, since the galactose oxidase treatment could be shortened to 10 min (8) without any effect on the test results, although the oxidation of a terminal galactose to the C-6 aldehyde usually requires 15–30 min at 37°C, while nonterminal galactose units are much less efficient substrates (10). Seeking an explanation to why colorectal mucus from patients with colorectal cancer developed a magenta colour directly upon addition of Schiff reagent, we first found that the component(s) of the mucous responsible for the colour development (referred to as a marker in this article) were soluble in lipophilic solvents. This suggested that the marker could not be a carbohydrate, which should be hydrophilic. We also found that asialofetuin (a suitable model glycoprotein because it contains T-antigen) does not undergo a change in colour when reacted with Schiff reagent, whether treated with galactose oxidase or not.

Since the marker reacted with Schiff reagent *directly*, we speculated that such a marker could become the basis of a simpler and more economical screening test (11) than the test involving galactose oxidase. The elimination of the oxidation step would reduce the costs of chemicals, equipment, and technician time. The first step toward this practical goal was to identify the chemical structure(s) of the marker(s). In this article we describe both the isolation and the identification of the chemical structure of the markers. Although the objective of this investigation has been the identification of the chemical structure of the markers, patient data gathered while collecting colorectal mucous in the process of the identification of the markers are presented (cf. Fig. 1 for examples). These data suggest that further epidemiologic investigations of the usefulness of the markers in a screening test for colorectal cancer are warranted.

Scheme 1. Chemical formulae of T-antigen before (**I**) and after (hypothesized **II**) oxidation with galactose oxidase, palmitic (**III**) and stearic (**IV**) aldehydes, and plasmalogens of ethanolamine (**V**) and choline (**VI**) types.



I



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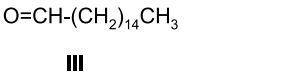
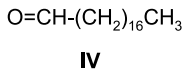
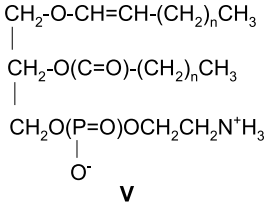


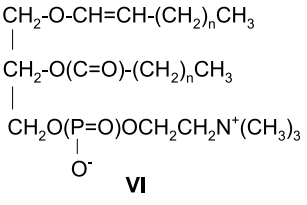
III



IV



V



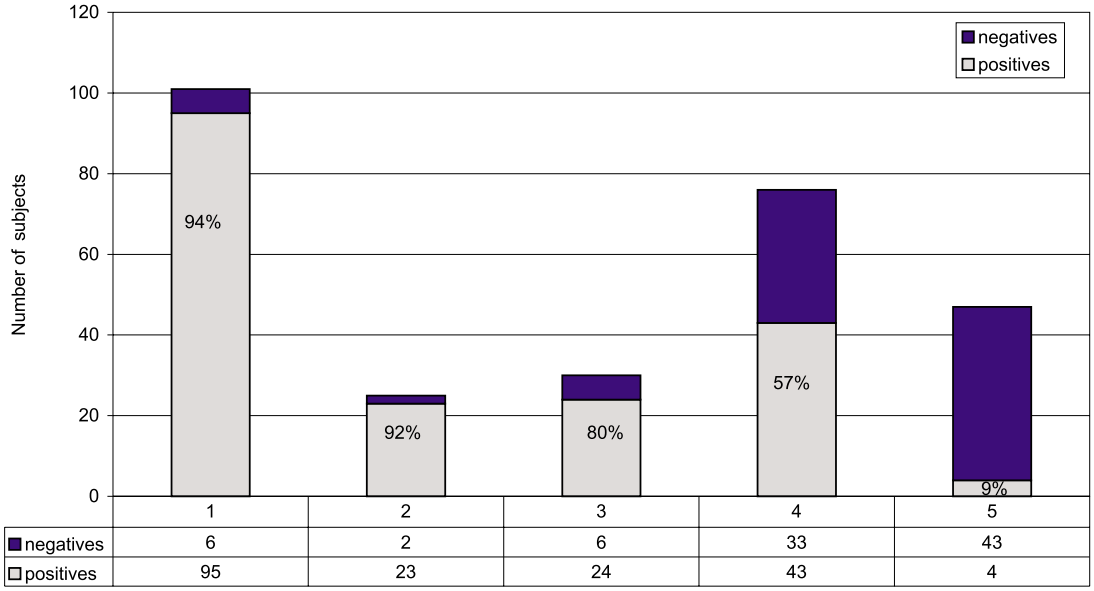
VI

Materials and methods

Materials and general procedures

Thin layer chromatography (TLC) was performed on silica gel 60 plates (thickness 0.25 mm) or preparative plates (thickness 1 mm). Analytical plates were visualized by spraying with 50% aq H_2SO_4 and heating to 200°C. Silica gel (200–400 mesh, Toronto Research Chemicals) was used for column chromatography. PE (brain extract) and PC (heart extract) plasmalogens were purchased from Doosan Serdary Research Laboratories (Toronto, Canada). Schiff reagent was prepared from *p*-rosaniline chloride (#1528, also basic fuchsin or magenta from Sigma Chemical Co.,

Fig. 1. Proportion of positives on five groups of subjects. Mucus examples were (1) from colectomy specimens from colorectal cancer patients obtained in several Toronto hospitals; (2) from individuals scheduled for colonoscopy at the Endoscopy suite, Wellesley Hospital, Toronto, subsequently diagnosed with cancer (mucus specimens were collected immediately before colonoscopy); (3) from individuals scheduled for colonoscopy at the Endoscopy suite, Wellesley Hospital, Toronto, subsequently diagnosed with large (larger than 1 cm in diameter) polyps (mucus specimens were collected immediately before colonoscopy); (4) from individuals scheduled for colonoscopy at the Endoscopy suite, Wellesley Hospital, Toronto, subsequently diagnosed with small (1 cm or smaller in diameter) polyps (mucus specimens were collected immediately before colonoscopy); (5) symptoms-free young adults (without any lavage performed before mucus collection). Note that colorectal cancer often, but not always, develops in polyps.



St. Louis, Mo.) or from basic fuchsin (ICN, Aurora, Ohio). Asialofetuin (A1908 and A4781) and galactose oxidase were purchased from Sigma. HPLC was performed on a variety of silica gel columns (5 μm, e.g., Hypersil) using a Beckman System Gold with a Beckman UV 166P detector or a Varex Evaporative Light Scattering Detector IIA. ^{1}H NMR spectra were recorded at 500 MHz with a Varian Unity spectrometer in the NMR spectroscopy laboratory of the Molecular Medicine Research Centre, University of Toronto. Spectra were obtained in $CDCl_3$, mixtures of $CDCl_3$–CD_3OD containing a trace of TMS (δ = 0 ppm) as the internal standard, or D_2O and are reported using a δ (ppm) scale. Mass spectra were recorded with VG Analytical ZAB-SE, SCIEX API III, or Voyager-Elite MALDI (Perseptive Biosystems) mass spectrometers in the mass spectrometry laboratory, Molecular Medicine Research Centre, University of Toronto. *O*-(2,3,4,5,6-Pentafluorobenzyl)oximes of aldehydes were analyzed by GC–CI(–)-MS (reagent gas: ammonia) using a VG-Trio 2A quadrupole mass spectrometer interfaced to a Hewlett-Packard 5890 Series II gas chromatograph at the Hospital for Sick Children, Toronto. Visible spectra were recorded with a Beckman DU 600 or a Varian CARY 300 Bio UV–Vis spectrometer (in solution or on the plates in transmittance mode). Reflectance scans were executed directly on the plates with a Varian CARY 50 spectrometer with remote reflectance probe.

Isolation of markers from surgical (colectomy) specimens

Mucous collection

Mucous was obtained from colectomy specimens from surgeries performed in operating theatres of several hospitals in Toronto, Ont., Canada. The colectomy specimens were washed with water to remove blood and fecal matter 15–20 min after surgery. Mucous was collected by gently scraping the surface with a small spatula. The scraped mucous was placed into small plastic vials and frozen. One colectomy specimen typically yielded 0.5–1.0 g of mucous; some specimens, however, were much larger. To determine whether the collected mucous gave a colour reaction with Schiff reagent, the frozen vials were allowed to thaw at room temperature for 60 min, and a small amount of mucous on the tip of a spatula was smeared on the support and tested as described in the following section.

Separation of mucous components

Mucous collected as above was pooled (66 g) and lyophilized for 24 h to give a semi-solid residue (6.0 g). This residue was consecutively extracted with hexane, ethylacetate, chloroform–methanol (2:1), modified Folch extraction (12), and water; the final residue after extractions was a gel-like solid (colonic mucin). Ethylacetate extract gave a weak colour reaction with Schiff reagent, while the chloroform–methanol extract gave a very strong colour reaction with Schiff reagent. Both extracts giving colour with Schiff reagent (i.e., positive) were combined and subjected to chromatography on a column of silica gel using solvents of increasing polarity: hexane, ethylacetate, chloroform, chloroform–methanol (with increasing contents of methanol), and methanol. Chloroform–methanol (7:2.5) afforded positively reacting fractions, which were combined and evaporated to dryness to give an amorphous residue containing phospholipids (36.6 mg), which reacted strongly positive with Schiff reagent (using conditions described in the

preceding paragraphs). This fraction was repeatedly chromatographed on a series of silica gel columns using chloroform with a gradient of methanol to give two highly positively reacting fractions, PL-1 and PL-2, which were further analyzed by ^{1}H NMR spectroscopy.

Testing mucous obtained in digital rectal examination

Mucous collection

Mucous samples were collected from individual human subjects by a physician during routine digital rectal examination using a gloved finger lubricated with MUKO or a similar lubricant that would not react with the Schiff reagent under the testing conditions. Informed consent of the tested individuals was obtained as per the protocol approved by the Ethics Committee, University of Toronto. Mucous collected on the gloved finger was smeared onto a support (e.g., glass fibre (Whatman GF) or polyester (polymacron, Dupont, Wilmington, Del.) fabric), showing through an aperture of 1.0–1.3 cm in diameter and fastened between two glass plates (7.5 × 7.5 cm) or sealed between two plastic plates (of microscope slide size). The plates with smeared mucous samples were sent to a laboratory for processing. If processed at the place of collection, the work-up should have started no earlier than 90 min after depositing the sample to obtain sufficient specimen adherence to the fabric. The plates carrying specimens could be stored for short periods of time at room temperature (several days) or stored frozen for prolonged periods of time (several months).

Treatment of mucous specimens with Schiff reagent

Plates carrying mucous specimens were placed in the holder part of a Wheaton glass staining dish (capacity 10 plates), immersed in phosphate buffer (0.1 M, pH 7.0), and washed by gentle agitation for 10 min. The holder was then transferred into a dish containing distilled water and gently agitated for 3 min. The aqueous wash was repeated; any excess water was allowed to drip for 10–15 min to render the plates and the holder visibly dry. The holder was then immersed into a dish containing Schiff reagent for 2 min under gentle agitation followed by three washes with distilled water (3 min each). The plates were removed from the holder and air dried. The pink-red-magenta colour (positive reaction) appeared almost immediately in some cases (or within a few minutes (20–25 min at the most)) after removal from the Schiff reagent.

Preparation of suitable Schiff reagent

Many variations of the reagent are described (6). The Schiff reagent used in this study was prepared as follows: *p*-rosaniline (0.4 g; also basic fuchsin, magenta) was added to distilled water (220 mL) at approximately 95–97°C. The mixture was stirred well, brought to boil for 5 min, cooled to 50°C, and filtered through doubly folded paper filter. Aqueous HCl (1 N, 34 mL) was added to the filtrate with stirring and was allowed to cool to room temperature. Sodium bisulfite (2.34 g) was added and the solution was stirred well — the flask was sealed with parafilm — and stored in the dark at room temperature for 4 days. To the resulting straw-coloured solution was added charcoal (NORIT; 300 mg); the mixture was vigorously stirred for 1.5 min, and the charcoal was removed by filtration. The resulting colourless solution was stored in a dark-glass bottle at 3–5°C.

Identification of compounds giving colour reaction with Schiff reagent

2,4-Dinitrophenylhydrazones of aldehydes from mucous

Mucous (4.27 g) obtained from a colectomy as described above and giving a colour reaction with Schiff reagent was spread with a spatula on five sheets (10 × 4 cm) of polyester fabric (polymacron) and allowed to dry for 1 h. The sheets were soaked in phosphate buffer (pH 7) for 20 min, then lifted from the buffer, washed with water until all the dark coloured material was removed and the sheets were slightly yellow, and air-dried. The sheets were then cut into smaller pieces (1 × 2 cm) and immersed into a solution of 2,4-dinitrophenylhydrazine hydrochloride (30 mL; prepared by dissolving 2,4-dinitrophenylhydrazine hydrochloride (0.25 g) in 6 N HCl (100 mL)) at room temperature for 30 min. The polyester cuttings were then removed from the reagent solution and washed with water until the eluant remained colourless; the deep-yellow 2,4-dinitrophenyl hydrazones were extracted from the cuttings by acetonitrile. Acetonitrile was removed on a rotary evaporator; ethylacetate (10 mL) was added and evaporated (to remove traces of water) to yield a dry yellow residue. The residue was dissolved in dry acetonitrile (1.5 mL) and applied on a silica gel 60 plate (20 × 20 cm; thickness 1 mm) in a single line; the plate was developed with a hexane – ethyl acetate solution (4:1). The yellow band at R_f 0.37 containing 2,4-dinitrophenylhydrazones was cut out and extracted with 10% MeOH in CH_2Cl_2; the solvents were evaporated to give a deeply yellow solid, which was subsequently analyzed by MS. Two major ions were $[M+H]^+$ (m/z = 421) and $[M+H]^+$ (m/z = 449). ^{1}H NMR ($CDCl_3$) confirmed the presence of 2,4-dinitrophenyl derivatives of long chain aldehydes: 0.88 (t, 3H, CH_3, J = 6.84 and 7.08 Hz), 1.25–1.42 (m, CH_2), 6.96 (t, CH=N), 7.53 (t, CH=N), 7.93 (d, H-6 (arom), J = 9.52 Hz), 7.96 (d, H-6 (arom), J = 9.52 Hz), 8.29 (dd, H-5 (arom), J = 3.18 and 9.76 Hz), 8.33 (dd, H-5 (arom), J = 3.17 and 9.52 Hz), 9.12 (d, H-3 (arom), J = 2.45 Hz), 9.14 (d, H-3 (arom), J = 2.45 Hz), 11.00 (s, NH), 11.18 (s, NH).

Two controls were applied: (1) the above procedure up to the chromatography was repeated with blank polyester sheets (no mucous deposited) — no reaction with 2,4-dinitrophenylhydrazine was detectable; and (2) the above procedure was repeated using mucus from colectomies that did not give a colour reaction with Schiff reagent — no reaction with 2,4-dinitrophenylhydrazine was detectable.

2,4-Dinitrophenylhydrazone of palmitic aldehyde from a plasmalogen

A mixture of PE plasmalogen (from brain extract; 10 mg in 2 mL $CHCl_3$), a solution of 2,4-dinitrophenylhydrazine hydrochloride (25 mL, prepared as above), and isooctane (20 mL) was stirred for 1 h at room temperature. The aqueous phase was separated and washed with isooctane; organic phases were combined and evaporated to dryness. The yellow residue was dissolved in dry acetonitrile (0.4 mL) and applied on a silica gel 60 plate (20 × 20 cm; thickness 0.2 mm) in a single line, and the plate was developed with

hexane – ethyl acetate (4:1). The yellow band containing 2,4-dinitrophenylhydrazone was cut out and extracted with 10% MeOH in CH_2Cl_2. Solvents were removed from the extract by evaporation to give a yellow amorphous solid, which was examined by MS. The major ion was $[M+H]^+$ (m/z = 421). ^{1}H NMR ($CDCl_3$; major isomer is *E* (*syn*); minor isomer is *Z* (*anti*)): 0.88 (t, 3H, CH_3, J = 6.84 and 7.08 Hz), 1.25–1.42 (m, 24H, CH_2), 1.61 (q, 2H, CH_2 β to CH, J = 7.33 Hz and J = 14.65 Hz, *E* isomer), 1.67 (q, 2H, CH_2 β to CH, J = 7.06 Hz and J = 15.38 Hz, *Z* isomer), 2.38 (ddd, 2H, CH_2 α to CH, J = 5.44, 7.33, and 12.94 Hz, *Z* isomer), 2.43 (ddd, 2H, CH_2 α to CH, J = 5.70, 7.57 Hz and J = 12.94 Hz, *E* isomer), 6.96 (t, 1H, CH=N, J = 5.37 and 5.62 Hz, *Z* isomer), 7.53 (t, 1H, CH=N, J = 5.37 and 5.13 Hz, *E* isomer), 7.93 (d, 1H, H-6 (arom), J = 9.52 Hz, *E* isomer), 7.96 (d, 1H, H-6 (arom), J = 9.52 Hz, *Z* isomer), 8.29 (dd, 1H, H-5 (arom), J = 3.18 and 9.76 Hz, *E* isomer), 8.33 (dd, 1H, H-5 (arom), J = 3.17 and 9.52 Hz, *Z* isomer), 9.12 (d, 1H, H-3 (arom), J = 2.45 Hz, *E* isomer), 9.14 (d, 1H, H-3 (arom), J = 2.46 Hz, *Z* isomer), 11.00 (s, 1H, NH, *E* isomer), 11.18 (s, 1H, NH, *Z* isomer).

Preparation of palmitic aldehyde (13)

A solution of diisobutylaluminum hydride (DIBAL-H) in toluene (1 M, 2.95 mL, 2 eq) was added dropwise over 5 min to a solution of methyl palmitate (0.4 g, 1.48 eq) in dry toluene (10 mL) cooled to –70°C. The reaction mixture was stirred for an additional 15 min, and excess reagent was decomposed by 2 N HCl (10 mL); the mixture was allowed to warm up to 0°C, and the aqueous layer was separated and extracted with ethyl acetate (2 × 10 mL). The combined organic extracts were washed with water (3 × 5 mL), dried over Na_2SO_4, and evaporated to dryness to give an amorphous solid containing both the aldehyde (60%) and starting ester (40%). ^{1}H NMR ($CDCl_3$) (aldehyde): 9.77 (d, $J_{1,2}$ = 1.95 Hz, CH_2CH=O), 2.42 (m, $J_{1,2}$ = 1.95 Hz, CH_2CH=O); (unreacted ester): 3.7 (s, CH_3OC[=O]-).

Preparation of 2,4-dinitrophenylhydrazone of palmitic aldehyde

The crude aldehyde (50 mg) dissolved in isooctane (25 mL) and a solution of 2,4-dinitrophenylhydrazine hydrochloride (25 mL, prepared as above) was stirred at room temperature for 1 h. After work-up (analogous to the work-up described for 2,4-dinitrophenylhydrazones of aldehydes from plasmalogens) and ensuing chromatography, a yellow amorphous solid was obtained. MS: $[M+H]^+$ m/z = 421. The chemical shifts in the ^{1}H NMR spectrum were identical with the shifts in the ^{1}H NMR spectrum of 2,4-dinitrophenyl hydrazone of stearic aldehyde (cf. below).

Preparation of stearic aldehyde

A 1 M solution of diisobutylaluminum hydride (DIBAL-H) in toluene (2.95 mL, 2 eq) was added dropwise over 5 min to a solution of methyl stearate (0.44 g, 1.48 eq) in dry toluene (10 mL) cooled to –70°C. The reaction mixture was stirred for an additional 15 min, and excess reagent was decomposed by 2 N HCl (10 mL); the mixture was allowed to warm up to 0°C, and the aqueous layer was separated and extracted with ethyl acetate (2 × 10 mL). The combined organic extracts were washed with water (3 × 5 mL), dried over Na_2SO_4, and evaporated to dryness to give an amorphous solid containing both the aldehyde (30%) and starting ester (70%). ^{1}H NMR ($CDCl_3$) (aldehyde): 9.77 (d, $J_{1,2}$ = 1.95 Hz, CH_2CH=O); 2.42 (m, $J_{1,2}$ = 1.95 Hz, CH_2CH=O); (unreacted ester): 3.7 (s, CH_3OC[=O]-).

Preparation of 2,4-dinitrophenylhydrazone of stearic aldehyde

A solution of the crude aldehyde (50 mg) in CH_2Cl_2 (4 mL) with added solution of 2,4-dinitrophenylhydrazine hydrochloride (26 mL, prepared as above) was stirred at room temperature overnight. After work-up (analogous to the work-up described for 2,4-dinitrophenylhydrazone of palmitic aldehyde) and following thin layer chromatography on silica gel, a yellow amorphous solid was obtained. MS: 449 ($[M+H]^+$). ^{1}H NMR ($CDCl_3$, major isomer: *E*, minor isomer: *Z*): 0.88 (t, 3H, CH_3, J = 6.84 and 7.08 Hz), 1.25–1.43 (m, 28H, CH_2), 1.61 (q, 2H, CH_2 β to CH, J = 7.31 Hz and J = 14.89 Hz, *E* isomer), 1.67 (q, 2H, CH_2 β to CH, J = 7.08 Hz and J = 15.65 Hz, *Z* isomer), 2.38 (ddd, 2H, CH_2 α to CH, J = 5.44, 7.33, and 12.94 Hz, *Z*-isomer), 2.43 (ddd, 2H, CH_2 α to CH, J = 5.68, 7.57 Hz and J = 12.94 Hz, *E* isomer), 6.96 (t, 1H, CH=N, J = 5.38 and 5.37 Hz, *Z* isomer), 7.53 (t, 1H, CH=N, J = 5.61 and 5.13 Hz, *E* isomer), 7.93 (d, 1H, H-6 (arom), J = 9.52 Hz, *E* isomer), 7.96 (d, 1H, H-6 (arom), J = 9.52 Hz, *Z* isomer), 8.29 (dd, 1H, H-5 (arom), J = 3.17 and 9.52 Hz, *E* isomer), 8.33 (dd, 1H, H-5 (arom), J = 3.17 and 9.52 Hz, *Z* isomer), 9.12 (d, 1H, H-3 (arom), J = 2.44 Hz, *E* isomer), 9.14 (d, 1H, H-3 (arom), J = 2.45 Hz, *Z* isomer), 11.00 (s, 1H, NH, *E* isomer), 11.18 (s, 1H, NH, *Z* isomer).

O-(2,3,4,5,6-Pentafluorobenzyl) oximes of aldehydes (14)

The above compounds were prepared from *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (250 µL of a 0.05 M solution in sodium acetate buffer, pH 5), added, in separate experiments, to the phospholipid mixtures PL-1 and PL-2 (cf. section "Separation of mucous components") as well as to authentic specimens of palmitic and stearic aldehydes (1 mg in 100 µL of water), vortexed for 1 min, and allowed to react for 30 min. Then 1 N HCl (10 µL) was added, and the reaction mixture was extracted three times with hexane (1 mL). The combined hexane extracts were dried over sodium sulfate and evaporated to dryness under a stream of nitrogen; the residue was redissolved in hexane (50 µL). This solution (1 µL injections) was used for GLC–MS identification of the aldehydes. Negative ion CI-MS (reagent gas: ammonia) was executed on a quadrupole mass spectrometer VG-Trio 2A interfaced with a Hewlett-Packard 6890 Series II GC using a DB-5 capillary column (length: 30 m, diameter: 0.25 µm), employing helium as the carrier gas at a flow rate of 1 mL min^{-1}, and with the temperature increasing from 60 to 310°C while maintaining the injection port temperature at 250°C (15).

Results

Aldehyde identification

Analytical chemists have been using Schiff reagent for decades to demonstrate that the aldehydic group is present in the examined samples. However, although the reagent is

very sensitive, it is extremely difficult to isolate the products of its reaction with aldehydes (6, 7). 2,4-Dinitrophenylhydrazine in a strongly acid solution, on the other hand, produces chemically stable 2,4-dinitrophenylhydrazones, which can be isolated and purified; often they can be obtained in a crystalline form. Employing modern spectroscopic methods, the chemical structure of such pure compounds can be determined with relative ease. As no aldehydic hydrogen *H*-C=O could be identified in the NMR spectra of the extracts from which 2,4-dinitrophenylhydrazones were prepared, the aldehydes had to be released from precursors during the treatment with Schiff reagent. Since both Schiff reagent and 2,4-dinitrophenylhydrazine operate in strongly acidic environments, it is reasonable to presume that the acidity of the reagents allowed for the release of the aldehydes from their precursors. Therefore, 2,4-dinitrophenylhydrazones (2,4-DNPH) are valuable derivatives for the identification of the structure of markers. Indeed, palmitic and stearic aldehydes were identified via their 2,4-dinitrophenylhydrazones as the aldehydes originating in colorectal mucous from cancer patients by the combination of MS and ^{1}H NMR spectroscopy. First, strong molecular ions were found in FAB-MS, which corresponded to the 2,4-dinitrophenylhydrazones of palmitic ($[M+H]^+$: m/z = 421) and stearic ($[M+H]^+$: m/z = 449) aldehydes. This finding was confirmed by ^{1}H NMR spectroscopy, by comparison of the NMR spectra of the 2,4-DNPH of aldehydes obtained from mucous fractions with synthetic authentic specimens, and by a further comparison with the previously published ^{1}H NMR data. As expected, two geometric isomers were present in all specimens of 2,4-DNPH of aldehydes and assigned as described in reference 16. The ^{1}H NMR chemical shifts of the 2,4-dinitrophenylhydrazones of palmitic and stearic aldehydes were found to be practically identical. These results indicated that the above aldehydes are the markers detected by Schiff reagent in colorectal mucous. The next question to be answered was: From what precursors were the aldehydes released?

During the chromatography of colorectal mucous, we observed that the fractions containing phospholipids produced the strongest colour with Schiff reagent. Since the only phospholipids that could release the long chain aliphatic aldehydes by treatment with acids are the plasmalogens, we therefore attempted to detect plasmalogens in the phospholipid fraction. To purify the plasmalogens, the phospholipid fraction was further rechromatographed on silica gel columns. Two well-separated groups of phospholipids (PL-1 and PL-2) were obtained. The first group contained ethanolamine and the other contained choline classes of phospholipids; both groups gave strong colouration on treatment with Schiff reagent. As these groups could not be further fractionated, we employed ^{1}H NMR spectroscopy to confirm the presence of the plasmalogens in these fractions (and consequently, to confirm the hypothesis that the aldehydes were released from the plasmalogens).

Plasmalogen identification

^{1}H NMR analysis (17) showed that both fractions were phospholipids with long fatty chain acids (*sn*-glycero-3-phosphoryl derivatives) based on ^{1}H resonances characteristic for glycerol and CH_2-groups of polar head groups and for aliphatic chains of fatty acid ester groups. The less polar fraction consisted of a mixture of phospholipids containing ethanolamine (PL-1), while the more polar fraction contained a mixture of phospholipids with choline (PL-2); neither of the two fractions was further separable by chromatography on silica gel. In the fraction PL-1, hydroxyls in the 2-position and partially in the 1-position of *sn*-glycerol were esterified with fatty acids. The ethanolamine ($-OCH_2CH_2NH_2$) moiety was identified by ^{1}H resonances at δ = 3.13 ppm (triplet) and at δ = 4.03 ppm (overlapping with H-3 of *sn*-glycerol). The presence of a signal of an α,β-unsaturated ether was confirmed through a doublet ($J_{1,2}$ = 6.1 Hz) at δ = 5.90 ppm, which was assigned to the vinylic proton O-C*H*=CH-. This proton was further coupled to a quartet at δ = 4.38 ppm and a multiplet at δ = 2.02 ppm in a COSY spectrum confirming the presence of the O-CH=CH-CH_2 moiety in the molecule. Other allylic CH_2 groups giving rise to signals at δ = 2.82–2.88 ppm were probably present in the olefinic chains of aliphatic acids. Two ^{1}H multiplets assigned to H-2 of the glycerol moiety centered at δ = 5.18 ppm and δ = 5.25 ppm suggested that this fraction contained two derivatives of ethanolamine-containing phospholipids. The integrations of the vinylic signal and the H-2 glycerol signal at δ = 5.18 ppm were of the same intensity (1:1). The estimate of the ratio (7:3) between the 1-alk-1′-enyl-2-acyl-*sn*-glycerophospholipids (= plasmalogens) and 1,2-diacyl-*sn*-glycerophospholipids was made on the basis of a comparison of the integration of signals for CH-2 of the glycerol moiety (cf. above).

In the more polar fraction PL-2, OH-2 and, partially, OH-1 in *sn*-glycerol were again esterified with fatty acids. The signals at δ = 3.24 ppm and δ = 3.40 ppm were identified as belonging to two different phospholipids containing a choline moiety (CH_2CH_2-N(CH_3)$_3$). As in the PL-1 fraction, this fraction contained an α,β-unsatured ether, which was confirmed through a doublet at δ = 5.90 ppm assigned to the vinylic proton O-C*H*=CH-. Two proton multiplets, centered at δ = 5.16 ppm and δ = 5.25 ppm and assigned to H-2 of the glycerol moiety, suggested that this fraction contained two derivatives of choline-containing phospholipids. The integration of the vinylic signal and the H-2 *sn*-glycerol signal at δ = 5.16 were again of the same intensity (1:1). The ratio between the 1-alk-1′-enyl-2-acyl-*sn*-glycerophospholipids (= plasmalogens) and 1,2-diacyl-*sn*-glycerophospholipids in PL-2 was estimated to be 4:1 by comparison of the integration of signals for CH-2 of the glycerol moiety (cf. above).

Stereochemistry of the glycerol moiety was not determined, although it was assumed that it does not differ from stereochemistry commonly found in natural phospholipids based on comparisons of the ^{1}H NMR spectra. Similarly, the geometry of the vinylic ether double bond in plasmalogens isolated from the colorectal mucus of cancer patients and plasmalogens from other natural sources was found to be identical (based on the identity of the coupling constant of the vinylic signal for ^{1}H at δ = 5.90 ppm).

The aldehydes released by hydrolysis of PL-1 and PL-2 were identified as stearaldehyde and palmitaldehyde by comparison with authentic specimens of *O*-(2,3,4,5,6-pentafluoro)benzyl oximes of the aldehydes using MS combined with GLC. These derivatives exhibited positive ions at *m*/*z*

435 ([M – H]), 415 ([M – 20]), and 350 ([M – 50]) — with [M – 20] predominating owing to the loss of HF (palmitaldehyde) — and at *m/z* 463 ([M – H]), 443 ([M – 20]), and 413 ([M – 50]), again with [M – 20] predominating, for stearaldehyde.

Structure of the marker

Thus, we have identified the markers as a mixture of long chain aliphatic aldehydes released from plasmalogens, predominantly palmitic (**III**) and stearic (**IV**) aldehydes (cf. Scheme 1). Plasmalogens are phospholipids of both the ethanolamine **V** and the choline **VI** types (cf. Scheme 1) in which the aldehydes are cryptic in the form of unsaturated ethers (18). A minor contribution from other aldehydes cannot be excluded; similarly, acetal phospholipids in which the aldehydes are bound as acetals to the C1–C2 of glycerol may be minor precursors. Because of the acid lability of unsaturated ether bonds, the aldehydes are released from plasmalogens under very mild acid conditions (19); therefore, it is not surprising that the aldehydes are released upon exposure to the strongly acidic Schiff reagent. The free aldehydes, on reaction with the *leuko* (colorless) form of the dye in Schiff reagent, provide shades of magenta colour considered characteristic of aldehydes (7, 8).

The lack of colour of the Schiff reagent depends critically on the presence, in sufficient concentration, of SO_2 and HCl in the reagent. Should this concentration decrease, the Schiff reagent may be partially restored to the original red colour of *p*-rosaniline. Therefore, depending on the aldehyde concentration, the shade of colour on the solid support may be observed as a mixture of red (20) and magenta (purple). The adduct of *p*-rosaniline–aldehyde (resulting from the reaction of an aldehyde with Schiff reagent) has a typical absorption >560 nm; therefore, the presence of such an absorption band in a reflectance spectrum should resolve the occasional colour identification problems (21). Schiff reagent further resorts to the original colour of *p*-rosaniline on treatment with an excess of basic substances, for instance, basic amino acids such as lysine, histidine, and arginine; nucleosides such as cytidine; or simple bases such as sodium carbonate. Thorough washing with water and a buffer solution ensures that all such result-altering components of mucous are removed from the specimens before treatment with the Schiff reagent.

Potential of the detection of marker aldehydes in a screening test for colorectal cancer

In the course of the marker identification studies it was necessary to use mucous from individuals whose disease status was known (usually determined by colonoscopy). It was also necessary to determine whether the treatment of the collected mucous with Schiff reagent resulted in colour formation (positive reaction). Therefore, rectal mucous was obtained from patients with colorectal cancer, from patients with various other pathological findings, and from individuals who had no complaints and thus were presumed to be disease free. It was also necessary to establish suitable conditions (as described in the Material and methods section) of the treatment of specimens, such as length of time for the reaction with the Schiff reagent, washings with buffers and water, different supports on which the treatment was performed, and the choice of a variant of Schiff reagent. Through these investigations we could confirm that nearly all mucus specimens from patients with colorectal cancer were positive. For instance, the aldehydes were detected in close to 94% of the mucus samples removed from colectomy specimens obtained in several Toronto hospitals from 101 patients diagnosed with colorectal cancer by colonoscopy (cf. Fig. 1). In rectal mucous specimens collected from colorectal cancer patients attending the Endoscopy suite of the Wellesley Hospital (after the lavage of the colon but before the colonoscopy) and in the rectal mucous of patients with polyps a high proportion of positive results was obtained (cf. Fig. 1). Similar results were obtained with mucous from patients before the lavage (data not shown). In symptom-free individuals (47 students of medicine, ages 20–29), the aldehydes were not detectable in mucus from 43 individuals. Mucous specimens were treated with Schiff reagent as described in the Materials and methods section.

Discussion

A number of clinical (8) and population studies on the mucous test employing the sequence galactose oxidase – Schiff reagent have shown (22) that the test has the potential (23) to become an alternative to the fecal occult blood test (FOBT) (24) as an efficaceous, simple, and economical screening tool for colorectal cancer (25). Some of the shortcomings of the galactose oxidase – Schiff reagent test appear to be due to the targeting of the incorrect carbohydrate marker, one which required repeated, extensive washings and treatments with enzymes (galactose oxidase and catalase), leading to the loss of the marker from test plates. Our finding — that the marker of the mucous test for colorectal cancer screening is of a lipid rather than of a carbohydrate character (including the disaccharide T-antigen) — makes it possible to correlate more accurately the presence of the marker with the disease and to develop a more reliable screening test for the disease. Significantly increased quantities of the plasmalogens in neoplasms of the large bowel (26, 27) and some other tissues (28, 29) were independently found by ^{31}P NMR spectroscopy. Since the amounts of both the ethanolamine and choline families of plasmalogens in neoplastic tissues appear to be significantly increased compared with disease-free tissues, it appears likely that it is a biochemical pathway leading to the unsaturated ether formation that has a connection with the neoplastic transformation.

Plasmalogens are a class of ubiquitous membrane phospholipids. Although the highest levels are normally found in tissues such as brain and heart, small concentrations have been reported in most other normal tissues, and small increases in plasmalogen levels in cancer cells have been previously observed (30, 31). Increased concentrations in the colon were ascribed to a combination of an increased level of the synthetic enzyme CTP – phosphocholine cytidyltransferase and a decreased concentration of hydrolytic enzymes, phospholipases C and D (32). However, biosynthesis of the "aldehydic moiety" of plasmalogens is varied (33, 34).

Presently, a connection between colorectal cancer and plasmalogens can only be hypothesized. For instance, plasmalogens, because of their effect on membrane fluidity, may

facilitate the cellular uptake of carcinogenic substances (35), e.g., of dietary (36) or microbial origin (37, 38). It is of interest to note that the majority of strict anaerobes contain detectable plasmalogens, while aerobic and facultative anaerobic bacteria lack plasmalogens (39). However, plasmalogen-like fecal mutagens (fecapentaenes, produced in the human colon by several anaerobes) seem to play no role in the etiology of human colon cancer (19).

An aldehyde-based test for colorectal cancer screening would be expected to have an advantage over fecal occult blood test (FOBT) and colonoscopy (for screening purposes only!). It could, potentially, uncover earlier stages of the neoplastic progression than both FOBT and colonoscopy because the latter two techniques target more developed cancer. Therefore, *the aldehyde test could detect cases not sufficiently developed to be detected by colonoscopy*. For instance, we have found several patients with strongly positive aldehyde mucus tests who were given a clean bill of health by colonoscopy (therefore they were rated as "false" positives). The same patients returned to the clinic within no later than 1–2 years with frank cancer. Given the lengthy progression of colon carcinogenesis, it is not unlikely that these individuals already harbored the disease at the time of the first aldehyde test. Since it is believed that the progression from early neoplastic changes to frank cancer may span decades (40), an earlier detection of the disease may lead to the realization of the hope that drugs could control and perhaps reverse the disease progression. Early results with non-steroid antiinflammatory drugs (e.g., aspirin) (41), inhibitors of cyclooxygenase-2 (COX-2) (42), and identification of molecular targets of these drugs (43) suggest that such hopes are not unrealistic. Equally encouraging is the prospect that the early neoplastic changes could be reverted, for example, through dietary or other lifestyle measures.

In summary, we believe that the identification of aldehydes from plasmalogens — rather than galactose-containing oligosaccharides — as the substances in colonic mucous that seem to correlate with colorectal cancer and that react with Schiff reagent, will make it possible to design meaningful epidemiologic studies that will confirm whether the aldehydes are indeed markers of colorectal cancer suitable for population screening.

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