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Reactivity of an anti-(human gastric carcinoma) monoclonal antibody with core-related peptides of gastrointestinal mucin

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Summary. A murine anti-(human gastric carcinoma) monoclonal antibody, GL-013 (IgG1), which reacts with a high-molecular-mass glycoprotein from colorectal tumour tissue [Yang and Price (1989) Anticancer Res 9: 1707], was examined for reactivity against a panel of purified and partially purified antigens associated with tumours of the gastrointestinal tract. These included carcinoembryonic antigen (CEA), normal cross-reacting antigen, Y-hapten glycoproteins, and perchloric acid extracts and glycolipid preparations from colorectal tumours. While the GL-013 antibody failed to bind to these antigens, it was found to react strongly with synthetic peptides with sequences based upon that reported for the protein core of a human gastrointestinal mucin [Barnd et al. (1989) Proc Natl Acad Sci USA 86: 7159; Gum et al. (1989) J Biol Chem 264: 6480]. In control tests, a series of other anti-(colorectal tumour) antibodies (IgG1 and IgG3), with broad reactivity towards gastrointestinal carcinomas, as well as an anti-CEA antibody, (IgG1) failed to react with the synthetic peptides. It is concluded that the anti-(gastric carcinoma) monoclonal antibody GL-013 binds to a threonine-rich peptide epitope expressed within the protein core of gastrointestinal mucins.

Key words: Monoclonal antibody – Antigenic peptides – Gastric carcinoma

Introduction

Rabbit antibodies have been raised against the chemically deglycosylated protein core of mucins isolated from human colon carcinoma LS174T xenografts grown in nude mice [7]. These antibodies were then employed to identify mucin cDNA clones in a small-intestine λg11 library and these clones contained 69- base-pair tandem repeats. The deduced consensus amino acid sequence of 23 residues for these repeats was determined to be high in threonine and proline and low in serine content. To what extent these mucins may be associated with tumours of the gastrointestinal tract is unknown and this requires investigation, since in other systems (notably in breast and ovarian cancer), epithelial mucins are expressed by tumours and they have been exploited as markers of malignant disease [9, 11, 13]. The amino acid sequence of gastrointestinal mucin protein repeat motif, however, differs entirely from that of the breast mucin repeat [1, 5, 7].

In the course of developing monoclonal antibodies against tumours of the human gastrointestinal tract, a number of antibodies have been produced that showed reactivity with malignant tissues and, to variable extents, with normal tissues as well. Even though the target antigens or epitopes for these had not been identified, it was considered likely that these antibodies would react with widely expressed gastrointestinal mucins. In order to test this proposal, a series of overlapping heptapeptides with sequences based upon that reported for the gastrointestinal mucin repeat motif – Pro-Thr-Thr-Thr-Pro-Ile-Thr-Thr-Thr-Thr-Thr-Val-Thr-Pro-Thr-Pro-Thr-Pro-Thr-Gly-Thr-Glu-Thr [1, 7] – were synthesised and tested for their capacity to bind the various monoclonal antibodies. Of the antibodies tested, one – GL-013, produced against human gastric carcinoma cells – was found to react with the mucin core peptides, and the minimum structure required for antibody binding involved the threonine-rich motif Ile-Thr-Thr-Thr-Thr-Thr-Val.

Materials and methods

Monoclonal antibodies. The antibody GL-013 (IgG1) was developed at the Cancer Research Institute, Shenyang, People's Republic of China, using cells of the P3NS1 myeloma as fusion partners for spleen cells from a BALB/c mouse immunised with dissociated gastric carcinoma (well-differentiated adenocarcinoma) cells isolated from a lymph node metastasis [20].

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The production of the monoclonal antibodies C365 (IgG1, anti-CEA), C161 (IgG1, anti-NCA/CEA), C14 (IgM, anti-Y-hapten) and NCRC-11 [IgM, anti-(breast carcinoma cells)] have previously been reported [17, 2, 4 respectively]. Monoclonal antibodies C154 (IgG1), C505/4 (IgG1), C692/23 (IgG3), C692/42 (IgG1) and C692/43 (IgG1) are a series of related reagents that are broadly reactive with human colorectal carcinoma cells and, to varying extents, with normal colorectal tissues, although their target antigens and/or epitopes remain to be identified.

Antigen preparations. GL-013-defined antigens were isolated from detergent (Nonidet P-40)-solubilised colorectal tumour subcellular membranes by immunoadsorbent chromatography using Sepharose-linked GL-013 antibodies [20]. Fractions eluted from the immunoadsorbent column with 100 mM diethylamine (pH 11.5) were tested for retention of GL-013-antibody-binding activity using a radioisotopic antiglobulin assay, and fractions with antigenic activity were dialysed against phosphate-buffered saline (PBS) before storage at –20° C.

Y-hapten-bearing glycoproteins were prepared from the sputum of a Y-hapten secreter using a C14 antibody immunoadsorbent column [15]. Carcinoembryonic antigen (CEA) (180 kDa) was purified from hepatic metastases from primary colonic adenocarcinoma [10] and the normal cross-reacting antigen (NCA; 60 kDa) was isolated from normal human spleen [14] and high-molecular-mass (>400 kDa) epithelial mucins were isolated from normal urine using an NCRC-11 antibody immunoadsorbent column [16].

Total lipid fractions from normal and tumour colorectal homogenates were prepared by chloroform/methanol extraction [12]. The dried extracts were reconstituted in PBS.

Radioisotopic antiglobulin test. Antigen preparations (purified antigens were at approximately 10 μg/ml in PBS containing 0.02% NaN_3) were added to 60-well Terasaki microtest plates (well capacity = 10 μl; Labtech Division, Miles Laboratories, Naperville, Ill., USA) at 10 μl/well. The antigen-containing plates were air-dried by overnight incubation at 37° C. The wells were washed four times with a washing buffer consisting of PBS containing 0.1% bovine serum albumin (BSA), 0.1% rabbit serum (RbS) and 0.02% NaN_3. During the final wash cycle, the wells were incubated with the washing buffer for at least 30 min to complete the blocking of non-specific binding adsorption sites. Hybridoma supernatants or purified monoclonal antibodies (at the saturating concentrations of 1–10 μg/ml in washing buffer), or washing buffer alone in negative controls, were added at 10 μl/well. After incubation for 1–2 h at room temperature, the wells were aspirated and washed four times. ^{125}I-labelled, affinity-purified F(ab')$_2$ fragments of rabbit anti-(mouse Ig) were added at approximately 10^5 cpm/10 μl/well (radioiodination of this reagent was performed using a chloramine T procedure [8] with 500 μCi ^{125}I/25 μg protein). Incubation was continued for 1–2 h at room temperature. The wells were aspirated, then washed six times, after which the radioactivity in each well was determined.

The non-specific binding of antibodies to 'PBS-coated' and 'BSA/RbS-blocked' wells was determined and the values obtained were subtracted from those determined with antigen-coated, BSA/RbS-blocked and antibody-treated wells.

Epitope mapping. Peptides, bound at the C terminus to polyethylene multi-pin supports, were synthesised according to the procedure of Geysen et al. [6] using an epitope mapping kit (Cambridge Research Biochemicals, Cambridge). Each heptapeptide was synthesised in duplicate. Two tetrapeptides, Pro-Leu-Ala-Gln and Gly-Leu-Ala-Gln, were also prepared so that an anti-(Pro-Leu-Ala-Gln) monoclonal antibody could be utilised as a positive control for the synthetic procedure. Peptides bound to their pin supports were tested for their capacity to bind various monoclonal antibodies using conventional enzyme-linked immunosorbent assay (ELISA) techniques. After each assay, bound antibodies were carefully disrupted from the pins by sonication in disruption buffer (1% sodium dodecyl sulphate, 0.1% 2-mercaptoethanol, 0.1 M NaH_2PO_4, adjusted to pH 7.2 with NaOH), followed by washing in water and hot methanol according to the manufacturer's recommendations.

Immunocytochemistry. Antibody binding to paraffin sections of gastrointestinal tissues and their tumours was examined by standard immunoperoxidase staining (detailed in [20]). Endogeneous peroxidase was inhibited by incubation with 0.3% H_2O_2 in 0.1% NaN_3, followed by blocking with human serum (10%), containing RbS (0.1%) and BSA (1%) in PBS. After incubating sections with monoclonal antibody, and washing, bound antibody was stained with peroxidase-conjugated rabbit anti-(mouse immunoglobulin) and diaminobenzidene, and sections were finally counterstained with haematoxylin and mounted.

Results

Epitope mapping

The peptide sequence of the 23-residue repeat motif of the core of gastrointestinal mucins has been reported as Pro-Thr-Thr-Thr-Pro-Ile-Thr-Thr-Thr-Thr-Thr-Val-Thr-Pro-Thr-Pro-Thr-Pro-Thr-Gly-Thr-Glu-Thr [1, 7]. A total of 24 heptapeptides (in duplicate) were synthesised, each covalently bound at its C terminus to the heads of polyethylene multi-pin supports according to the procedure of Geysen et al. [6]. These peptides overlapped by 1 amino acid such that the overlapping heptapeptides covered a single 23-residue repeat and 7 amino acids of an adjacent repeat.

The following monoclonal antibodies and human sera, in the order shown, were examined for their capacity to bind to the peptides on their solid-phase supports: (1) C505/4, (2) C692/43, (3) C154, (4) C692/42, (5) C692/23, (6) anti-RTA, (7) GL-013, (8) C365.

The anti-RTA antiserum (sample 6, at a dilution of 1/300) was from an ovarian cancer patient treated with an RTA (ricin A chain) antibody immunotoxin. Although the test with this serum was performed as a negative control for other studies, it also serves as a further control in the present investigation. All antibodies were tested as hybridoma tissue-culture supernatants (containing 10–25 μg/ml IgG) or as purified antibody (at 10 μg/ml). While definitive information concerning target antigens for the first six monoclonal antibodies (from C505/4 to GL-013) is generally lacking, their broad reactivity with tumour and normal tissues in standard immunohistological tests suggested that they might react with gastrointestinal mucins associated with colorectal and gastric tumours.

None of these antibodies, with the exception of GL-013, reacted with any of the tethered peptides. As shown in Fig. 1, the anti-(human gastric carcinoma) monoclonal antibody, GL-013 reacted with three of the peptides and showed maximum binding to the threonine-rich peptide Ile-Thr-Thr-Thr-Thr-Thr-Val. In all tests, an anti-(Pro-Leu-Ala-Gln) monoclonal antibody was tested against the immobilised peptide Pro-Leu-Ala-Gln and Gly-Leu-Ala-Gln, and absorbances for the positive and negative controls in the ELISA assays were always greater than 0.5 against Pro-Leu-Ala-Gln and less than 0.1 with Gly-Leu-Ala-Gln peptides. Only absorbances of 0.1 or greater were taken as representing a positive antibody-binding reaction.

The profile of reactivity of the GL-013 antibody, at a concentration of 10 μg/ml, with immobilised peptides is illustrated in Fig. 1. Equivalent profiles were obtained with the antibody examined at concentrations up to 50 μg/ml, and the GL-013 antibody was tested against the peptides in

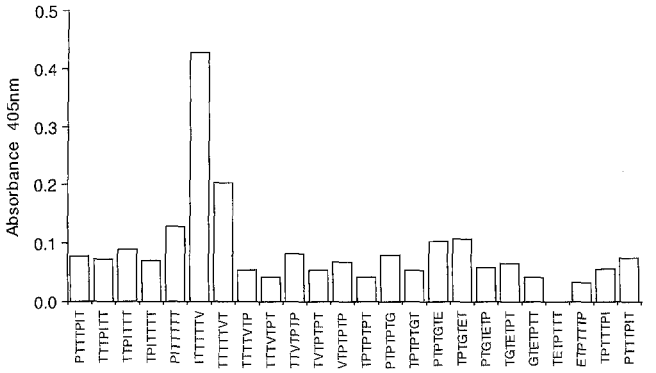

Fig. 1. Epitope-mapping assay. Monoclonal antibody, GL-013 (at 10 μg/ml) was tested for its binding to the synthetic heptapeptides shown using an enzyme-linked immunosorbent assay. The absorbance values represent the mean of values obtained with duplicate pins. With all positive signals (absorbance ≥0.1) little variation (approximately ± 5% of the mean value) was observed in the replicate determinations for each peptide. The single-letter symbols for amino acids are used in this figure (i.e. P, Pro; T, Thr; I, Ile; V, Val; G, Gly; E, Glu)

four separate assays. It is thus concluded that the GL-013 antibody reacts with a threonine-rich epitope expressed in the protein core of gastrointestinal mucins. The minimum sequence of amino acids common to those peptides reactive with the GL-013 antibody (i.e. peptides Pro-Ile-Thr-Thr-Thr-Thr-Thr, Ile-Thr-Thr-Thr-Thr-Thr-Val and Thr-Thr-Thr-Thr-Thr-Val-Thr; Fig. 1) is the sequence of 5 Thr residues so that this could be considered to represent the minimum epitope sequence for the antibody GL-013. However, the marked reduction in binding of GL-013 to the peptides Pro-Ile-Thr-Thr-Thr-Thr-Thr and Thr-Thr-Thr-Thr-Thr-Val-Thr, as compared to that with Ile-Thr-Thr-Thr-Thr-Thr-Val, suggests that Ile and Val residues, flanking the region of 5 Thr residues, are involved to some extent in the epitope for the GL-013 antibody.

Reactivity of GL-013 antibody with purified antigens

Previous studies with GL-013 indicated that this antibody reacted with a high-molecular-mass component (>200 kDa) as assessed in sodium dodecyl sulphate/polyacrylamide gel electrophoresis and immunoblotting tests [20]. It was further determined that affinity-purified antigen (isolated using an immobilised GL-013 antibody column) retained GL-013-antibody-binding activity after treatment with neuraminidase and collagenase, and it was only sensitive to proteolytic degradation with pronase, trypsin and papain at the highest concentrations of enzyme tested (100 μg/ml). Antigenic activity was not retained after treatment with $HClO_4$.

As shown in Table 1, the GL-013 antibody reacted strongly with affinity-purified antigen, but it failed to react with CEA or the normal cross-reacting antigen, NCA, from human spleen. Both of these antigens are found in $HClO_4$ extracts of colonic carcinoma (Table 1) although GL-013 failed to react with this extract. GL-013 was also unreactive with Y-hapten glycoproteins (>200 kDa), which bind the antibody C14 (Table 1) and antibodies of the 692 series). Furthermore, GL-013 was unreactive with glycolipid extracts of normal and malignant colon, with which the antibody C505 reacts. A preparation of urinary epithelial mucin (>400 kDa) also failed to bind the GL-013 antibody.

Table 1. Reactivity of monoclonal antibodies with various tumour-associated-antigen preparations

Target antigen[a]	Binding of monoclonal antibody (mean cpm ± SD) to target antigen					
	GL-013	C365	C161	C14	C505/4	NCRC-11
GL-013 antigen (>200 kDa)	5182 ±182					
CEA (180 kDa)	23 ±29	6211 ±454				
NCA (60 kDa)	−64 ±67		6396 ±332			
$HClO_4$ extract of colon Carcinoma tissue	63 ±31	6618 ±185	9778 ±307			
Y-hapten-bearing glycoproteins	−60 ±76			3902 ±535		
Normal colonic glycolipids	60 ±63				761 ±122	
Colorectal tumour glycolipids	178 ±32				3782 ±197	
Epithelial mucin (>400 kDa)	48 ±100					5547 ±380

[a] CEA, carcinoembryonic antigen; NCA, normal cross-reacting antigen

Immunocytochemistry

Examples of the distribution of the target antigen for GL-013 antibodies are shown in Fig. 2a, b. Most colorectal and gastric carcinomas stain with the GL-013 antibody [20] and Fig. 2a shows the intense staining of a poorly differentiated gastric carcinoma, which appears to be both intracellular and surface-associated. GL-013 antibody reacted mainly with normal epithelia of stomach and colorectal mucosae, and distinct staining was obtained with goblet cells (Fig. 2b), a major location for the production of gastrointestinal mucin.

Discussion

In the search for antigens-associated with malignant disease, it has become apparent that certain components of normal tissues may be be over- or abnormally produced in malignant cells and that these may be exploited as useful tumour markers. This is most notable with the high-molecular-mass epithelial mucins associated with specialised glandular epithelia and secreted by breast carcinomas into the circulation. Amounts of circulating mucin in breast carcinoma patients very much reflect tumour burden, and the response to therapy is matched by corresponding

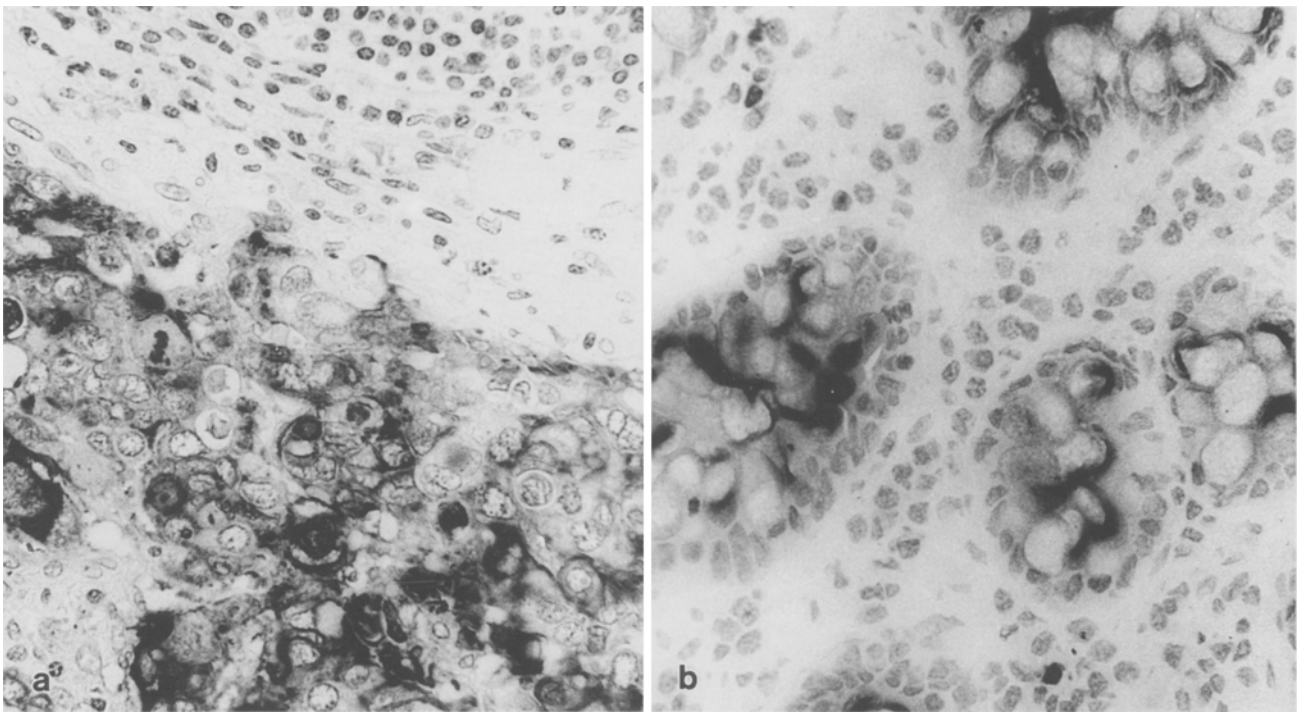

Fig. 2 a. Indirect immunoperoxidase staining of tissues using the GL-013 monoclonal antibody. **a** Intense, but heterogeneous staining of a specimen from a poorly differentiated gastric carcinoma, which is adjacent to unstained normal tissue and lymphoid cells towards the top of the figure. **b** Strong staining of goblet cells in normal colonic mucosae

movements in marker levels [9, 11, 13, 18]. The appearance of this mucin in the serum of cancer patients is considered to reflect disorganisation of tissue architecture in the breast as well as losses in the functional polarity in the malignant cell. Thus, secreted mucins normally produced for extracorporeal transport, gain access to the circulation in the cancer patient.

The sequence of 20 amino acids comprising the repeat motif forming the protein core of the epithelial mucins associated with breast carcinomas [5] differs entirely from the sequence of 23 amino acids deduced for the gastrointestinal mucin core [1, 7]. However, both classes of mucin bear similarities in their overall properties, being extensively glycosylated, high-molecular-mass glycoproteins. Indeed, both the breast mucins and gastrointestinal mucins exhibit a genetically controlled polymorphism, which in the case of the breast mucins has been demonstrated as a length polymorphism [5, 19]. Unlike the mammary mucins, few antibodies have been produced against the gastrointestinal mucin, and the GL-013 antibody appears to be among the first described that reacts with its protein core. Previous tests with GL-013 indicated that it displayed little preferential reactivity with tumours of the gastrointestinal tract, although clearly this is a reagent that requires evaluation for its capacity to measure circulating mucins in patients with gastrointestinal cancer. In addition, the GL-013 antibody may be employed in the preparation of immunoadsorbent columns for the isolation of mucins from tumours of the gastrointestinal tract. Aberrant glycosylation of tumour mucins or the action of tumour-associated glycosidases or glycosyl transferases may generate determinants in tumour mucins, which may further be exploited as tumour markers. Alternatively, by analogy with the breast-carcinoma-associated mucins, premature termination of glycosylation in tumours may expose determinants in the protein core with greater accessibility to antibodies [3] so that these may be then be exploited as markers of malignant cells.

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