Carbohydrate Research, 213 (1991) 47–57
Elsevier Science Publishers B.V., Amsterdam

Lactose-binding lectin expression in human colorectal carcinomas. Relation to tumor progression*,†

Reuben Lotan, Yoshifumi Matsushita, David Ohannesian, Dunia Carralero, David M. Ota, Karen R. Cleary, Garth L. Nicolson, and Tatsuro Irimura

Departments of Tumor Biology, General Surgery, and Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 (U.S.A.)

(Received January 17th, 1990; accepted for publication, March 19th, 1990)

ABSTRACT

Lactose-binding lectins having M_r values of ~14 000 (L-14.5) and ~35 000 Da have been found in a variety of vertebrate tissues, including normal intestine and colon, and in several types of tumors such as colon carcinomas. To determine the clinical relevance of such lectins in human colon cancer, specimens from 46 patients with colorectal carcinoma of identified Dukes' stages were selected and analyzed for the presence and amount of lactose-binding lectins by immunoblotting using a polyclonal, rabbit anti-lectin antibody followed by binding of ^{125}I-labeled anti-rabbit IgG. The amount of a lectin having an M_r value of ~31 000 Da (L-31) varied among the specimens. The levels of L-31 lectin in colorectal cancer specimens from primary tumors of patients with distant metastases (Dukes' stage D) were significantly higher than were those from patients without detectable metastases (Dukes' stages B1 and B2). In contrast, among the various specimens the variation in the level of the L-14.5 lectin was smaller, and there was no correlation between the amount of this lectin and cancer stage. Immunohistochemical staining of thin sections of colorectal tumor specimens using antibodies specific for either L-31 or L-14.5 lectin revealed that the two were located at different places, the L-31 lectin primarily within the cytoplasm of carcinoma cells, and the L-14.5 lectin associated with secreted material. These results indicated that the relative amount of the L-31 lectin increases as the colorectal cancer progresses to a more malignant stage.

INTRODUCTION

Adenocarcinomas of the colon and rectum constitute the second most prevalent type of cancer and the second major cause of cancer mortality in the Western World. Metastases already exist in some distant organs in 30% of patients at the time that colorectal cancer is diagnosed, and of patients apparently cured by surgery, 20–30% eventually develop recurrent metastatic cancer that leads to death.

The presence or absence of metastases is the most critical factor in determining a colorectal cancer patient's prognosis[1]. The development of sensitive diagnostic methods to identify patients at risk for development of metastases should improve prognosis[2], and the ability to determine which patients are at a high risk for recurrence after surgical

* This article is dedicated to Professors Toshiaki Osawa and Nathan Sharon who were doctoral thesis advisors for Tatsuro Irimura and Reuben Lotan, respectively.

† This study was supported by grants from the National Foundation for Cancer Research, The Council for Tobacco Research-USA, Inc. (No. 2546), the National Cancer Institute (CA-39319 and CA-50231), and The Texas Advanced Technology Program (No. 1549).

0008-6215/91/$ 03.50

removal of the primary tumor should provide critical information to medical oncologists[1].

A prerequisite for this approach is the identification of specific cellular and biochemical markers that change as premalignant colorectal lesions progress to metastatic tumors. These markers could also enhance the predictability of the current Dukes' classification for increasing malignancy of colon cancers[3,4], as well as estimate the prevalence of highly metastatic cells within a primary tumor. Based on this concept, we have been searching for cell surface and extracellular molecules associated with metastatic colorectal carcinoma in humans and have demonstrated that the expression of several mucin-like high-M_r glycoproteins correlated positively or negatively with the progression of colorectal carcinoma to the metastatic phenotype[5–10].

The expression of cell surface and secreted glycoconjugates, including mucins, glycoproteins, glycolipids, and blood-group-associated carbohydrate antigens, has been found to change during normal differentiation and after malignant transformation; some of these components are designated as oncodevelopmental antigens[11,12]. Several reports strongly suggested that complex carbohydrates play an important role in determining the malignant behavior of colorectal carcinoma and that such molecules might be promising biochemical markers of progression of this type of cancer[13–17].

Plant lectins have been widely used in the study of glycoconjugate structure, localization, and function in various tissues[18–20], including human normal, premalignant, and malignant colon tissues[5,7,21–24]. The presence of D-galactosyl and poly(*N*-acetyllactosamine)-containing oligosaccharides in the mucins and glycoproteins of normal and malignant colorectal tissues has been established by use of lectins[11,12,21–25]. Glycoconjugates containing terminal or penultimate D-galactose units may play a role in cellular interactions by serving as complementary molecules for endogenous lactose-binding proteins[26–29]. Such lectins have been found in the normal intestine of chickens[30–32], rats and mice[33,34], and rabbits[35], as well as in human colonic mucosa and colon carcinomas[36–40]. Lactose-binding lectins bind to blood-group antigens. For example, an $M_r \sim 29\,000$* lectin (L-29) from human lung exhibited a 30-fold higher affinity for blood group A related structures, including some derived from mucins, than for lactose. In contrast, an L-14.5 lectin from the same tissue had a lower affinity for the blood group A carbohydrate than for lactose[41]. The L-14.5 lectin from bovine heart was able to bind blood group H and B related carbohydrate structures[42]. Both the L-14.5 and the L-29 lectins from human lung had a higher affinity for poly(lactosamino)glycan chains than for lactose, but the L-29 lectin had a higher affinity than did the L-14.5 lectin[41].

As recently suggested by Leffler *et al.*[33], we refer to the D-galactosyl binding lectins as lactose-binding lectins, since D-galactose is a much poorer lectin ligand than is lactose, and we report herein the presence and amounts of lactose-binding lectins in colorectal carcinoma specimens to determine the clinical significance of lectin expression.

* All M_r values are expressed in dalton units.

EXPERIMENTAL

Surgical specimens and colorectal cancer cell line. — Fresh human tissues from 46 primary colorectal carcinomas and 3 liver metastases from colorectal carcinoma were collected from patients undergoing surgical resection at The University of Texas M. D. Anderson Cancer Center. The primary tumors were staged according to the Astler–Coller modification[4] of Dukes' classification system[3]. A surgical specimen from a poorly to moderately differentiated, mucin-producing adenocarcinoma in the right colon of a 74-year-old man and a normal colon specimen taken at autopsy from a 69-year-old man, who died of atherosclerotic coronary heart disease, were provided by Ms. K. C. Sexton (Cooperative Human Tissue Network, Tissue Procurement Service, The University of Alabama at Birmingham, AL). Human placenta was obtained from a local hospital. All the above-named tissue samples were stored frozen at −70°.

The human KM12P cell line, derived from a surgical specimen of a Dukes' B2 primary colorectal carcinoma[43], was provided by Dr. I. J. Fidler (The University of Texas M. D. Anderson Cancer Center, Houston, TX). The cells were grown in plastic tissue-culture dishes (Corning Glass, Corning, NY) in a 1:1 mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F12 medium (Grand Island Biological Co., Gibco, Grand Island, NY), supplemented with nonessential amino acids and vitamins (Gibco), 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), and antibiotics. The cells were grown at 37° in a temperature-controlled, humidified incubator in an atmosphere of 5% air and 95% CO_2.

Purification of lactose-binding lectins by affinity chromatography. — Lactose-binding lectins, obtained from KM12P cells, normal colon tissue, colorectal carcinoma specimens, and normal human placenta, were purified by affinity chromatography using immobilized asialofetuin as described previously[44] as follows. The normal colon tissue (100 g wet weight) and colon carcinoma tissue (1.5 g wet weight) were minced into small fragments and extracted by sonication in a 1:2 (v/w) extraction solution consisting of 4mM β-mercaptoethanol; 2mM ethylenediaminetetraacetic acid in calcium- and magnesium-free phosphate-buffered saline, pH 7.2 (MEPBS); and 0.3M lactose. The KM12P cell pellet was extracted by homogenization in the aforementioned extraction solution. Human placenta (400 g wet weight) was extracted as described by Couraud *et al.*[45]. A 100 000*g* supernatant fraction of each extract was dialyzed against the extraction solution without lactose (MEPBS), and applied onto an affinity column consisting of asialofetuin bound covalently to Affi-Gel 10 (Bio-Rad, Richmond, CA). After the unbound material had been washed out with MEPBS, the bound material was eluted with 0.3M lactose in MEPBS. Samples of the lactose-eluted material were subjected to poly(acrylamide)gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE), and the gels were stained with Coomassie Brilliant Blue.

Anti-lectin antibodies. — Antibodies prepared against a mixture of L-14.5 and L-19 lectins from bovine lung[46], which have been shown to cross-react with human lectins having similar M_r[47], were a gift from Dr. S. H. Barondes (University of California, San Francisco, CA). These antibodies were used for the immunoblotting

analyses. Antibodies against mouse 3T3 (M_r ~35000)lectin[48] (a gift from Dr. J. L. Wang, Michigan State University, East Lansing, MI), which had been found[49] to cross-react with human L-31 lectin, and antibodies prepared by us against the L-14.5 lectin from human placenta (see below) were used for the immunohistochemical localization of the lectins in sections of colorectal carcinoma specimens.

Human placenta lectin, purified by affinity chromatography as described above, was subjected to preparative SDS–PAGE in slab gels, and strips containing the L-14.5 lectin were excised from the gels and homogenized. Antibodies against human placenta lectin were prepared as described by Couraud *et al.*[45]. An initial immunization of rabbits with purified lectin (~100 μg) in complete Freund's adjuvant, injected intradermally at multiple sites, was followed by subcutaneous booster injections at two-week intervals with a similar amount of purified lectin emulsified in incomplete Freund's adjuvant. The specificity of the antibodies was verified by immunoblotting as described below. The serum collected after the fourth booster was used to prepare an IgG fraction by DEAE-Affi-Gel Blue chromatography as described by the manufacturer (Bio-Rad). A solution containing 50 μg/mL of protein was stored at −70°.

Immunoblotting analysis of lactose-binding lectins. — Tumor tissues were minced into small pieces (<0.5 mm) and solubilized by mixing 50-mg portions with an extraction buffer (0.5 mL) containing 0.5% Nonidet P-40, 0.25M sucrose, 0.05mM $CaCl_2$, and 0.01mM phenylmethylsulfonyl fluoride in 5mM Tris·HCl buffer, pH 7.3, for 18 h on ice[7]. The mixtures were centrifuged at 12000*g* for 5 min and the supernatant fractions were mixed with concentrated sample buffer. Samples (100 μg of protein) in sample buffer (35 μL) were subjected to SDS–PAGE in 13% poly(acrylamide) gels as described elsewhere[50]. After electrophoresis, the gels were placed against a nitrocellulose filter (0.1 μm, Micron Separation Inc., Westboro, MA), and the proteins were transferred electrophoretically at 150 V for 2 h. The nitrocellulose papers were soaked overnight at 4° in a saturation buffer consisting of 3% bovine serum albumin, 0.9% NACl, and 0.02% NaN_3 in 10mM Tris·HCl buffer, pH 7.4, and then incubated at 23° for 2 h in a 1:100 dilution (in the saturation buffer) of an antiserum prepared against a mixture of purified L-14.5 and L-29 lectins from bovine lung[46]. The nitrocellulose papers were then washed with 0.9% NaCl in 10mM Tris·HCl buffer, pH 7.4 (washing buffer), for 1 h at 23°, and incubated further for 2 h at 23° in a solution containing 1.5×10^6 c.p.m. of ^{125}I-labeled goat anti-rabbit IgG antibodies (0.47 MBq/μg, ICN Radiochemicals, Irvine, CA), in 10 mL of saturation buffer. Subsequently the nitrocellulose papers were washed for 1.5 h with a washing buffer that was changed every 15 min, dried, and placed against X-ray films for autoradiography.

To obtain quantitative results, the autoradiograms were placed on top of the nitrocellulose filters, and the lectin bands were excised. The amount of radioactivity (^{125}I-goat anti-rabbit IgG) associated with each band was determined with a gamma counter. Each slab gel included a lane with an extract prepared from 1×10^5 murine RAW117 lymphosarcoma cells, which contain L-14.5 and L-35 lactose-binding lectins; these lanes served as standards for comparison among different gels. Immunoblotting experiments with serially-diluted lymphosarcoma extracts indicated that the bound

radioactivity was proportional to the amount of lactose-binding lectins in the range that included extracts from 1×10^5 cells. The amount of radioactivity bound to lectin bands ($M_r \sim 14\,500$ and $M_r \sim 31\,000$) on the nitrocellulose paper was divided by the radioactivity bound to the L-14.5 and the L-35 lectins of the lymphosarcoma cells, respectively. The amount of radioactivity bound to these standard lectin bands was designed as 1 unit. The significance of the differences among lectin levels in specimens from different Dukes' stages was determined by analysis of variance.

Immunohistochemical localization of lactose-binding lectins in tissue section. — Immunohistochemical staining methods were described previously[5,23]. Briefly, serial sections of surgical specimens fixed in buffered formalin and embedded in paraffin were deparaffinized and treated with 0.03% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity. The sections were rehydrated and washed with Dulbecco's phosphate buffered saline (DPBS), then incubated with 1% bovine serum albumin (BSA, RIA grade) dissolved in DPBS for 2 h at room temperature, or overnight at 4°. The sections were incubated with an antiserum specific for the L-31 lectin (diluted 1:300) or with an IgG fraction (50 μg/mL) of an antiserum specific for the L-14.5 lectin (diluted to 1:200) in 1% BSA dissolved in DPBS for 16 h at 4°. The sections were washed several times with DPBS and treated with biotinylated goat anti-rabbit IgG for 1 h. After several washings with DPBS, the sections were further incubated with solutions of avidin–biotinylated-horseradish-peroxidase complex (ABC staining kit purchased from Vector Laboratories, Burlingame, CA) for 1 h at room temperature. After several more washes with DPBS, the sections were incubated with a peroxidase-substrate solution prepared by mixing 0.4% 3-amino-1-ethylcarbazole in *N,N*-dimethylformamide (1 mL; Sigma), 20mM sodium acetate buffer (100mL, pH 5.2), and 30% H_2O_2 (70 μL). The sections were then counterstained briefly with Meyer's hematoxylin and mounted with gelatin–glycerin mounting agent.

RESULTS AND DISCUSSION

Purification of lactose-binding lectins from normal colon, colon carcinoma tumor specimen, and cultured cells by affinity chromatography. — Affinity chromatography on immobilized asialofetuin of extracts obtained without detergents from normal colon resulted in the purification of a ~L-14.5 lectin (Fig. 1, C). In contrast, the same procedure performed on an extract from a surgical specimen of colon adenocarcinoma resulted in the purification of several lactose-binding proteins having apparent M_r values of ~14 500, ~22 000, ~31 000, and ~33 000 (Fig. 1, B). Interestingly, only an L-31 lectin was purified from an extract of cultured KM12P colon carcinoma cells (Fig. 1, A). These results suggested that the higher-M_r lectins are more prevalent in malignant colon cells than in normal colon tissue; they suggested also the possibility that the L-14.5 lectin detected in the surgical specimen of colon cancer may derive from normal cells contained within the tumor mass or that some colon cancer cells may produce an L-14.5 lectin *in vivo*.

In a previous study[39], affinity chromatography of salt extracts from a primary

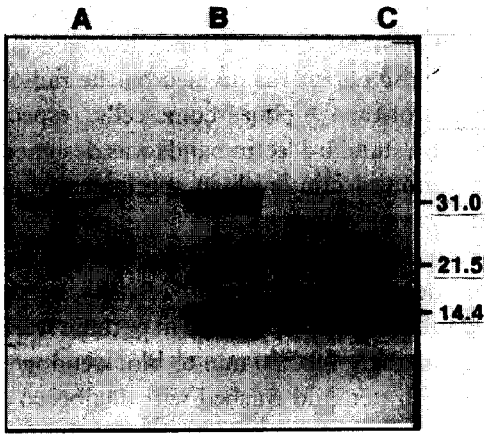

Fig. 1. Analysis of lactose-binding lectins purified by affinity chromatography using immobilized asialofetuin. Samples of concentrated peak fractions, eluted with lactose from the affinity column, were analyzed by SDS–PAGE in 13% gels and the proteins were stained with Coomassie Brilliant Blue: (A) Lectin from extracts of a cultured human KM12P colon carcinoma cell line; (B) lectins from an extract of a surgical specimen of a colon adenocarcinoma; and (C) lectin from an extract of a normal colon. The numbers on the right indicate the migration in the same slab gel of standard molecular-weight markers: 14 400 (hen egg white lysozyme), 21 500 (soybean trypsin inhibitor), and 31 000 (bovine carbonic anhydrase).

colon carcinoma on immobilized lactose detected lectins having M_r ~14 000 and ~64 000, but none were detected by affinity chromatography on immobilized asialofetuin. In contrast, affinity chromatography on immobilized asialofetuin of extracts from two other primary colon carcinoma specimens detected a lectin having M_r ~29 000 in one of the extracts and lectins having M_r ~26 000 and ~29 000 in the other[40]. These results indicated that heterogeneity exists among different primary colon carcinomas in their expression of lactose-binding lectins. Furthermore, differences were noted between lectins extracted from primary tumors and those extracted from metastases; lectins having M_r ~26 000 and ~54 000 were detected in a liver metastasis by affinity chromatography on immobilized asialofetuin, and lectins having M_r ~14 000, ~15 000, ~54 000, and ~66 000 were found in extracts from colon carcinoma metastases to liver by affinity chromatography on immobilized lactose. Normal human liver contains only the M_r ~14 000 and ~54 000 lectins[39].

Affinity chromatography requires a relatively large amount (gram quantities) of tissue to detect lactose-binding lectins[37–40]; Therefore, this technique is not applicable for the analysis of a large number of clinical specimens. For this reason, we used immunoblotting procedures that can be performed on much smaller tissue samples (milligram quantities).

Immunoblot analysis of lactose-binding lectins in specimens of colorectal carcinoma. — The presence of lactose-specific lectins in extracts from 49 colorectal carcinoma surgical specimens (46 primary tumors and 3 liver metastases) was analyzed by immunoblotting using specific antibodies. These antibodies recognize three lactose-binding lectins, *i.e.*, L-14.5 and L-31 in human cells, and a lectin having M_r ~34 000 (lectin L-34)

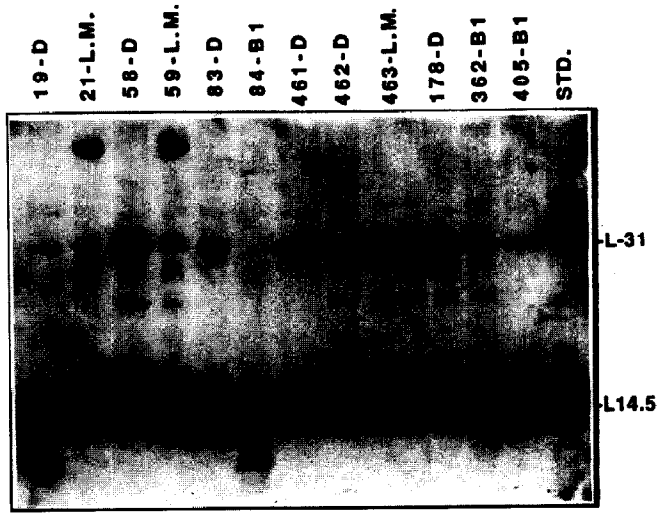

Fig. 2. Immunoblot analysis of lectin expression in colorectal carcinoma specimens. Twelve specimens were subjected to SDS–PAGE in 14% gels, electrophoretic transfer to nitrocellulose filters, and localization of lectin by successive incubation of the filters with antilectin antibodies and radio-iodinated anti-rabbit antibody. The filters were placed against an X-ray film, and the resulting autoradiogram is presented. The numbers and letters on top indicate the specimen number and the Dukes' stage of the specimen, respectively; L. M., liver metastasis; and STD., an extract prepared from mouse lymphosarcoma, which served as a standard for quantitation of antibody binding.

in murine cells[44,46–48]. The results of immunoblotting studies of 12 colorectal carcinoma samples are shown in Fig. 2. All these samples contained the L-14.5 lectin and most contained the L-31 lectin. Some samples included additional bands of lectin having M_r ~17 000, ~18 000, ~26 000, and ~46 000. The highest M_r band was noted in 2 of 3 liver metastases. Some samples contained a doublet of lectins having M_r ~31 000 and ~34 000, whereas most had only the L-31 lectin band. The relative amount of L-31 lectin varied among the various specimens. The finding of several lactose-binding lectins in a single specimen is compatible with the recent report of the presence of multiple lactose-binding lectins in rat and mouse intestine[33], and in human lung[41].

An analysis of the results after quantitation of lectin bands in all of the tumor samples demonstrated that the amount of L-31 lectin varied among the specimens, with 30% of specimens containing low levels of L-31 (<0.2 normalized units), 47% containing intermediate levels (0.2–0.6 normalized units), and 23% containing high levels (>0.6 normalized units). Fig. 3a demonstrates that the amounts of L-31 lectin in colorectal cancer specimens from advanced malignancies (Dukes' stage D) were significantly (P = 0.0005 by analysis of variance) higher than those in specimens from less advanced malignancies (Dukes' stages B1 and B2). In contrast, variation among specimens in the level of L-14.5 lectin was smaller, with the majority of specimens (94%) containing high amounts of lectin, and with no correlation between the amount of L-14.5 lectin and the stage of the cancer (Fig. 3b).

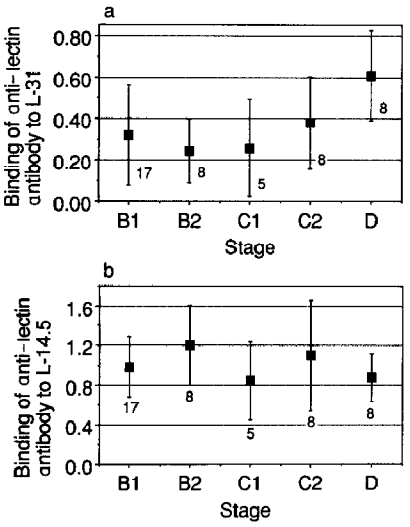

Fig. 3. Correlation between the levels of L-31 (a) and L-14.5 lectin (b) and the stage of colorectal primary carcinoma. The stages were determined by the Astler–Coller modification of Dukes' classification[4]. The analysis is based on results from 46 tumor samples analyzed by immunoblotting and quantitated as described in the Experimental section. The values correspond to the mean ± SD. The number of various specimens analyzed for each stage are indicated.

Immunohistochemical localization of the lectins in thin sections of colorectal tumor specimens. — Some colon tumor samples contain infiltrating normal host cells and connective tissue elements, which may contain one or both lectins. Furthermore, lectin expression within the tumor may be restricted to distinct subpopulations of carcinoma cells because intratumoral heterogeneity is often encountered in the analysis of metastasis-related cellular markers. It was important, therefore, to supplement the biochemical results described above with immunohistochemical studies to determine the localization of these lectins. Immunohistochemical staining of several thin sections of colon carcinoma specimens using antibodies specific for either L-31 or L-14.5 lectin (Fig. 4) revealed different locatizations for each of the two lectins. Anti-L-31 lectin antibody bound to carcinoma cells in a fairly homogeneous fashion within a tumor, primarily in the cytoplasm of the cells (Fig. 4A). In contrast, there was almost no staining of carcinoma cells within the same tumor with anti-L-14.5 lectin antibody, but positive staining was observed associated with secretory products (Fig. 4B). The anti-L-31 lectin antibody was bound to the pericellular region of normal colonic epithelium, and the anti-L-14.5 lectin antibody was bound occasionally to the surface of epithelial cells in the lower crypt (data not shown).

Previous studies have demonstrated that a chicken L-14.5 lectin (CLL-II) is located within the secretory granules of mucus-secreting goblet cells in normal chicken intestine, with labeling along the mucosal surface where the lectin is presumably associated with secreted mucin[30]. Studies on lectin location using a similar immunohistochemical technique in 24 mammary carcinomas showed that the L-14.5 lectin was

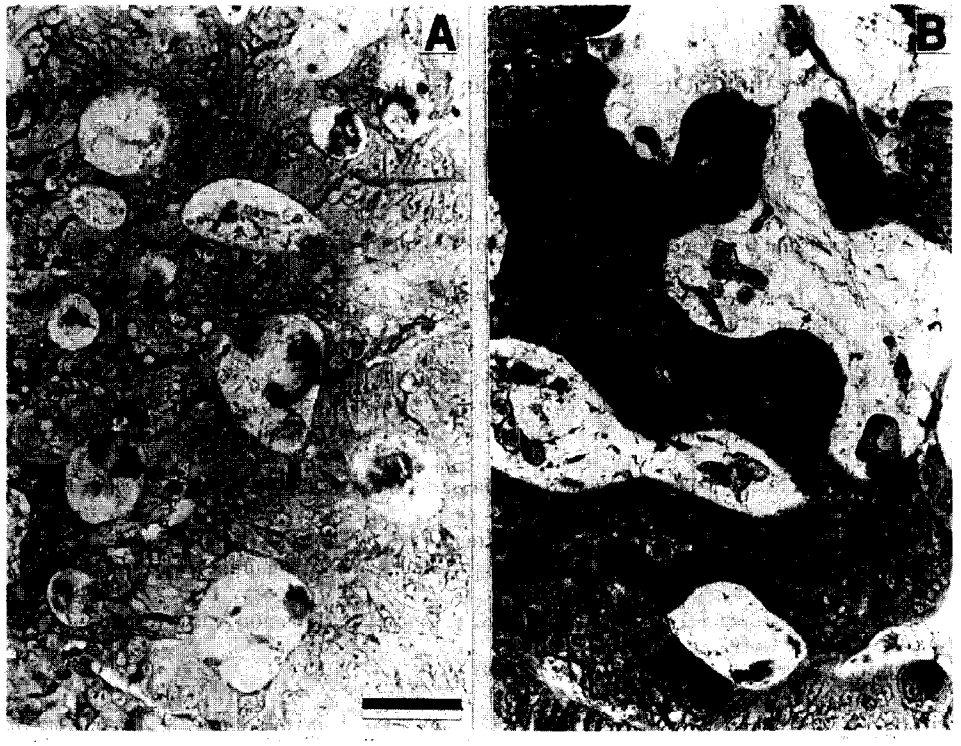

Fig. 4. Immunohistochemical staining of thin sections of colorectal carcinoma specimens with (A) anti-L-31 and (B) anti-L-14.5 lectin antibodies. The sections were taken from a surgical specimen of a colon adenocarcinoma, which was classified as stage D according to the Astler–Coller modification of Dukes' classification. Bound antibodies were visualized by use of biotinylated anti-rabbit IgG and avidin–biotinyl-peroxidase complex, and 3-aminoethylcarbazole. A very brief hematoxylin counterstain was performed. Both sections were photographed under the same magnification. The bar represents 50 μm.

expressed by few tumor cells, whereas L-29 lectin (probably the homolog of the L-31 lectin) was found in nearly all tumors. The staining for both lectins was localized primarily in the cytoplasm of the cells; however, some staining of the L-29 lectin was found in cell nuclei[38].

The present study is the first comparison of lactose-binding lectins in a large number of colorectal carcinoma specimens. It has enabled us to establish a correlation between the amount of L-31 lectin and colorectal cancer progression. Of interest is that previous studies with cultured murine cell lines demonstrated a correlation between the level of murine L-34 lectin (the counterpart of human L-31) and malignancy[27–51]. The present study and previous reports indicate that colorectal cancer cells contain lactose-binding lectins, and D-galactosyl- and poly(lactosamine)-containing glycoconjugates. However, additional studies are required to determine (a) whether these complementary molecules actually interact with the malignant cells and (b) what role, if any, the L-31 lectin plays in the pathogenesis of colorectal cancer progression and metastasis. We are currently conducting patient follow-up studies to determine whether the L-31 lectin can be used as a prognostic marker for colorectal cancer metastasis.

ACKNOWLEDGEMENTS

The authors thank Drs. S. Barondes and John L. Wang for a generous gift of anti-lectin sera.

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