Lectin binding properties of cells from serous effusion and peritoneal washing specimens

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SUMMARY The diagnostic value of staining cells from serous effusion and peritoneal washing specimens with a panel of four lectins was investigated and the results compared with those achieved with polyclonal anti-carcinoembryonic antigen (anti-CEA) sera. Cell blocks from 42 pleural effusions, 25 peritoneal effusions, and 14 peritoneal washing specimens were stained with Con A (D-mannosyl, D-glucosyl), WGA (n-acetyl glucosamine), UEA-1 (L-fucose), SBA (n-acetyl galactosamine) and anti-CEA. Con A and WGA were not useful in discriminating mesothelial cells from adenocarcinomas. In contrast, UEA-1 and SBA binding was present in 30 of 46 (65%) and in 31 of 46 (67%), respectively, of adenocarcinomas tested, but negative in 21 cases with reactive mesothelial cells, 10 cases with benign mesothelial cells, and one case of mesothelioma. All mesothelial cells were also negative for CEA, but 24 of 46 (52%) of adenocarcinomas were positive. All three cases of lymphoma were negative with UEA-1, SBA, and anti-CEA.

UEA-1 and SBA lectins identified a higher proportion of metastatic adenocarcinomas than CEA and stained most cases of adenocarcinomas metastatic from sites which usually fail to express CEA. Combination of staining results for UEA-1, SBA, and anti-CEA produced a test with high sensitivity and specificity, identifying 40 of 46 (87%) of adenocarcinomas tested, with no false positive results. It is concluded that UEA-1 and SBA staining of effusion specimens warrants further study, and may provide a useful adjunct to CEA staining.

The cytological diagnosis, using morphological criteria, of serous effusion and peritoneal washing specimens has long been recognised as posing particular difficulties. This is because, in some cases, it is not possible to differentiate adequately between metastatic carcinoma cells, reactive (hyperplastic) mesothelial cells, and malignant mesothelial cells.^{1,2} Scanty malignant cells may also be overlooked in specimens stained by conventional Papanicolaou and Giemsa stains. These problems have been circumvented to some extent by immunocytochemical staining, but these techniques also have limitations, due to the variable expression of antigens by tumours or their common expression by both cells of mesothelial origin and metastatic carcinoma cells.³⁻⁶ Nevertheless, recent interest in immunochemical staining of histological and cytological specimens has refocused attention on cellular carbohydrate chemistry, in particular on those changes occurring at the plasma membrane of neoplastic cells. Indeed, most monoclonal antibodies directed against surface antigens identify carbohydrate determinants (epitopes).⁷

The expression of neo-carbohydrate epitopes by neoplastic cells is the result of aberrant glycosylation of cellular glycoconjugates (glycoproteins and glycolipids). This aberrant glycosylation has been identified in several tumour types by the use of lectins.⁷⁻¹⁰

Lectins are protein and glycoproteins derived from animals and plants which possess high specificity for monosaccharides and oligosaccharides. The binding of lectins to specific sugars occurs in a manner similar to an antibody-antigen reaction,¹¹ and this reaction may be visualised with several enzyme detection systems. Although lectin binding has been studied using histological specimens to investigate several diseases, there are very few studies on the role of these substances in cytology.

Material and methods

The material consisted of 42 pleural effusion, 25 peritoneal effusion, and 14 peritoneal washing specimens submitted for cytological examination to the cytology department of this hospital. Specimens were prepared for routine cytological diagnosis by

cyto centrifugation and smearing of cellular pellets. These preparations were fixed in 95% ethanol for staining by the Papanicolaou method and air-dried for Giemsa staining. Material from each specimen was also prepared as cell blocks using the method of Heyderman and Brown¹² after fixation in methacarn (60% methanol, 30% chloroform, 10% acetic acid). Sections 5 µm thick were then cut for staining with haematoxylin and eosin, anti-CEA, and lectins. The following four lectins were used: Concanavalin A (Con A), wheat germ agglutinin (WGA), *Ulex europaeus* agglutinin I (UEA-I), and soybean agglutinin (SBA). Cell blocks selected for this study all contained cell populations of one predominant cell type, with peritoneal washing specimens providing a source of easily identifiable benign mesothelial cells arranged in sheets. Cytological diagnoses of specimens, confirmed by histological examinations, are listed in table 1. Thirty one cases were diagnosed as benign and 50 cases as malignant. Patient data and histology reports were obtained from patient and histopathology records.

CEA STAINING

Cell block sections were stained using the peroxidase-anti-peroxidase (PAP) method,¹³ with a rabbit polyclonal antiserum to CEA (Dako, Denmark). All dilutions were made in 10% normal swine serum (NSS). Incubations were performed at room temperature. The method was as follows:

- 1 Dewax and rehydrate sections.
- 2 Immerse in 6% hydrogen peroxide and 8% methanol in phosphate buffered saline (PBS), pH 7.4, for 15 minutes to quench endogenous peroxidase.

- 3 Wash in running tap water.
- 4 Incubate with 10% NSS for 20 minutes.
- 5 Drain and incubate with rabbit anti-CEA, diluted 1/60, for 30 minutes.
- 6 Wash in PBS for one minute × 3.
- 7 Incubate with swine anti-rabbit serum, diluted 1/400, for 30 minutes.
- 8 Wash in PBS one minute × 3.
- 9 Incubate with rabbit peroxidase-anti-horseradish peroxidase, diluted 1/40, for 30 minutes.
- 11 Develop substrate reaction in 0.02% 3-3' tetrahydrochloride diaminobenzidine (DAB) (Sigma, St Louis, Missouri, USA), with 0.015% hydrogen peroxide in PBS for 3 minutes.
- 12 Wash in tap water and counterstain with haematoxylin. Positive controls for CEA staining consisted of methacarn fixed, paraffin wax embedded sections of fetal colon. Negative controls were performed by omitting the incubation with anti-CEA.

LECTIN STAINING

Lectins were selected for their sugar specificity (table 2). Biotinylated lectins and sugars were purchased from Sigma Chemicals. All incubations were performed at room temperature. The staining protocol was as follows:

- 1 Dewax and rehydrate sections.
- 2 Immerse in 6% hydrogen peroxide and 8% methanol in PBS for 15 minutes.
- 3 Wash in running tap water.
- 4 Incubate with 2% bovine serum albumen (BSA) in Hank's balanced salt solution (Commonwealth Serum Laboratory, Melbourne), for 20 minutes.
- 5 Drain and incubate with biotinylated lectin diluted in 10% BSA in Hanks's balanced salt solution (Con A: 40 µg/ml, WGA, UEA-I and SBA: 50 µg/ml) for 60 minutes.
- 6 Wash in PBS for one minute × 3.
- 7 Incubate with streptavidin-biotin-peroxidase (SPB) complex (Amersham, Birmingham) diluted 1/400 in PBS for 30 minutes.
- 8 Wash in PBS for one minute × 3.
- 9 Develop substrate reaction with 0.02% DAB and 0.01% hydrogen peroxide in PBS for three minutes.
- 10 Wash in tap water and counterstain with haematoxylin.

Negative controls were performed by: (i) omitting

Table 1 Cytological diagnoses of serous effusion and peritoneal washing specimens

Diagnosis	Pleural fluid	Peritoneal fluid	Peritoneal washings	Total
Benign mesothelial cells	—	—	10	10
Reactive mesothelial cells	16	5	—	21
Mesothelioma	—	1	—	1
Adenocarcinoma	24	18	4	46
Lymphoma	2	1	—	3
Total	42	25	14	81

Table 2 Lectin specification

Lectin	Source	Sugar specificity	Inhibitory sugar
Con A	<i>Concanavalin ensiformis</i> (Jack bean)	α-D-mannosyl α-D-glucosyl	Methylmannopyranoside
WGA	<i>Triticum vulgaris</i> (Wheat germ)	N-acetyl-β-D-glucosaminyl residues N-acetyl-β-D-glucosamine oligomers	N-acetyl glucosamine
UEA-I	<i>Ulex europaeus</i> (gorse)	α-L-fucose	α-L-fucose
SBA	<i>Glycine max</i> (Soybean)	N-acetyl-D-galactosamine	N-acetyl-D-galactosamine

incubation with lectins; (ii) omitting incubation with SBP complex; and (iii) incubating lectins with their inhibitory sugar (0.02M in either PBS or in 2% BSA in HBSS) for 60 minutes before staining. As a positive control, sections of normal human skin, fixed in methacarn and embedded in paraffin wax, were also stained with each batch, as previous trials had shown positive staining with all four lectins in this tissue.

After staining with anti-CEA and lectins slides were examined for the characteristic brown reaction product.

Results

Lectin binding and CEA staining results are summarised in table 3. Specimens were assessed as positive if at least 10% of cells were stained. Although some variation was noted in the intensity of cellular staining, all cases could be clearly identified as either positive or negative. Background staining was rarely present and always considerably weaker than the staining present in positive cells.

CON A

Benign and malignant epithelial cells in all specimens stained positively with Con A. Benign leucocytes also exhibited Con A binding, but variability was noted in the staining of benign lymphocytes, which stained inconsistently or negatively in some cases. One case of lymphoma also failed to stain. In contrast, macrophages, adenocarcinoma cells, and mesothelial cells always stained positively. All cell types exhibited cytoplasmic staining.

WGA

Wheat germ agglutinin failed to stain consistently cells of any type. Variation was also noted in the distribution of staining on epithelial cells. In most positive specimens most staining was localised on the plasma membrane. In these cases cytoplasmic staining was either weak or absent. Other positive specimens exhibited cytoplasmic staining alone. These staining patterns were not correlated with specimen diagnosis or with the primary sites of metastatic adenocarcinomas. Leucocytes also varied in their ability to bind WGA. Lymphocytes and polymorphs exhibited only

occasional cytoplasmic staining, but macrophages were usually positive with strong plasma membrane staining and occasional cytoplasmic staining. No lymphoma cells examined stained.

UEA-1

Staining with UEA-1 was negative for all benign, reactive, and malignant mesothelial cells examined. In contrast 30 of 46 (65%) of adenocarcinomas examined stained positively. All known gastrointestinal tract tumours, all lung adenocarcinomas, and six of 10 (60%) of known ovarian tumours were positive (table 4). All benign leucocytes and lymphoma cells were negative. Cells positive with UEA-1 exhibited cytoplasmic staining (fig 1).

SBA

Mesothelial cells also failed to bind SBA. Adenocarcinoma cells, however, stained positively in 31 of 46 (67%) of cases. Adenocarcinomas known to be metastatic from the gastrointestinal tract all stained positively, as did most known lung, ovarian, and endometrial adenocarcinomas. Leucocytes usually failed to stain, but macrophages were occasionally positive in both benign and malignant specimens. Lymphoma cells were uniformly negative. In all positive cases some degree of cytoplasmic staining was present, but in 12 of 31 positive cases strong staining was also present on the plasma membrane of adenocarcinoma cells (fig 2). These staining patterns were not correlated with the primary sites of adenocarcinomas.

ANTI-CEA

CEA was not detected in any benign, reactive, or malignant mesothelial cells, but positive staining was present in 24 of 46 (52%) of adenocarcinomas. Although all known lung, and most gastrointestinal tract adenocarcinomas, were positive for CEA, most ovarian and endometrial tumours did not stain. Macrophages and neutrophils were often weakly positive, due to cross reaction of the anti-CEA sera with normal antigens present on these cells,¹⁴ and possibly due to residual endogenous peroxidase. Cells positive for CEA always exhibited cytoplasmic staining.

Table 3 Staining results with lectins and anti-CEA

Diagnosis	Con A No positive/ No tested	WGA No positive/ No tested	UEA-1 No positive/ No tested	SBA No positive/ No tested	Anti-CEA No positive/ No tested
Benign mesothelial cells	10/10	7/10	0/10	0/10	0/10
Reactive mesothelial cells	21/21	11/21	0/21	0/21	0/21
Mesothelioma	1/1	1/1	0/1	0/1	0/1
Adenocarcinoma	46/46	34/46	30/46	31/46	24/46
Lymphoma	2/3	0/3	0/3	0/3	0/3

Table 4 Primary sites of metastatic adenocarcinomas and their staining reactions with UEA-I, SBA, and anti-CEA

Primary site	UEA-I (No positive/No tested)	SBA (No positive/No tested)	anti-CEA (No positive/No tested)	Combined results UEA/SBA/anti-CEA (No positive/No tested)
Breast	3/9	5/9	4/9	7/9
Ovary	6/10	8/10	2/10	8/10
Endometrium	1/4	3/4	1/4	3/4
Fallopian tube	0/1	0/1	1/1	1/1
Lung	4/4	3/4	4/4	4/4
Stomach	1/1	0/1	0/1	1/1
Colon	1/1	1/1	1/1	1/1
Rectum	1/1	1/1	1/1	1/1
Pancreas	1/1	1/1	1/1	1/1
Liver	1/1	1/1	1/1	1/1
Unknown*	11/13	8/13	8/13	12/13
Total per cent positive	65%	67%	52%	87%

*Cases listed as unknown were those in which no histological record of primary tumour could be found.

Fig 1 Adenocarcinoma cells staining positively with UEA-I. (SBP method; haematoxylin counterstain.)

Fig 2 Breast adenocarcinoma cells staining positively with SBA. Strong plasma membrane labelling is present in some cells. Background leucocytes are negative. (SBP method; haematoxylin counterstain.)

Discussion

The results indicate that UEA-1 and SBA can be useful in differentiating between adenocarcinoma cells and mesothelial cells in serous effusions and peritoneal washing specimens. Staining was positive with UEA-1 and SBA in 65% and 67%, respectively, of adenocarcinomas tested, and negative in all mesothelial cells. Occasional macrophages were observed to stain positively with SBA, but these were rare and easily identified on the basis of morphology and arrangement. These lectins were effective in labelling metastatic adenocarcinoma cells from a wide range of primary sites, and both UEA-1 and SBA were more sensitive markers of adenocarcinoma than CEA.

Carcinoembryonic antigen has been widely used to assist the cytological diagnosis of effusions. Although CEA has high specificity, it has only moderate sensitivity, being expressed in 50–66% of adenocarcinomas metastatic to the pleural and peritoneal cavities.^{3–5} In this series CEA produced no false positive results, but only identified 24 of 46 (52%) of the adenocarcinomas tested. Adenocarcinomas of the endometrium and the ovary, other than mucinous cystadenocarcinomas, rarely express CEA,^{15,16} and most specimens with malignant cells from these sites were negative in this series. In contrast, UEA-1 and SBA were effective in labelling most adenocarcinomas from these sites. Thus a combination of the results obtained with UEA-1, SBA, and anti-CEA (table 4) produced a test with both high specificity (100%) and sensitivity 40 of 46 (87%) for metastatic adenocarcinoma in serous effusions and peritoneal washings.

In contrast, Con A and WGA were not useful in differentiating between the cell types present in these specimens. Examining whole cells from 22 fluid specimens, Lubinski found the pattern of fluorescein-labelled Con A binding helpful in identifying mesothelial cell, macrophages, and adenocarcinoma cells if specimens were labelled unfixed.¹⁷ All cell types, however, were found to stain in a similar pattern after glutaraldehyde fixation. The methacarn fixed cells examined in the present study also exhibited identical Con A binding patterns for all cell types. Although UEA-1 has blood group O specificity, and most epithelial cells express antigens of the ABO series,^{18,19} mesothelial cells were consistently negative with this lectin, even though strong positivity of erythrocytes was observed in some cases. No reports on UEA-1 or SBA binding to mesothelium seem to have been published, and none of the published photographs of UEA-1 or SBA stained tissues, studied by the author, contain identifiable mesothelium. Further study is thus needed to determine why these lectins do not bind to mesothelial cells. Caution must also be exercised in interpreting the results of lectin binding at a cellular

level. Lectin binding to free, simple sugar, as in controls, does not adequately reflect lectin-carbohydrate interactions at a cellular level. Fixation,²⁰ the configuration of adjacent or nearby sugars,²¹ and detection methods are all known to affect the observed strength of lectin binding.

The inability of SBA and UEA-1 to bind to mesothelial cells with this technique may be of diagnostic importance. Lectin staining is technically easy to perform and interpret and the costs are comparable with staining for CEA. Continued investigation of UEA-1 and SBA staining is clearly warranted to characterise fully their cellular specificity and to determine their diagnostic value in the examination of serous effusion specimens.

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