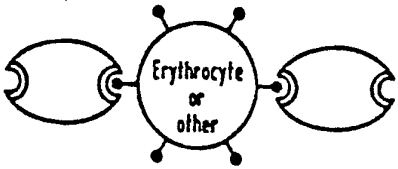
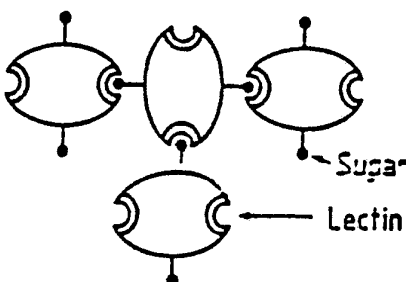




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(54) Title: SPECIFIC CARBOHYDRATE-BINDING PROTEINS (LECTINS) OF MAMMALIAN TUMOR CELLS		
A  Heterotypic aggregation B  Homotypic aggregation		
(57) Abstract Carbohydrate-binding proteins (lectins) of mammalian tumor cells and processes for their preparation. These lec- tins, the corresponding carbohydrates and the corresponding monoclonal antibodies are suitable for rapid, reliable and precise differential diagnosis of tumors and for the production of pharmaceutical compositions for the treatment of tu- mors.		

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Title

Specific carbohydrate-binding proteins (lectins) of mammalian tumor cells.

Technical field of the invention

This invention relates to carbohydrate-binding proteins (lectins) of mammalian tumor cells which specifically recognize and bind to carbohydrate molecules and to methods of isolating these lectins from mammalian tumor cells.

Background art

The designation "lectin" is derived from the property of certain proteins to "select" (i.e. recognize) specific carbohydrate structures and to form a lectin-carbohydrate complex.

Lectins can be defined as follows:

- the recognition of carbohydrates is highly specific and thus comparable to the antigen-specificity of antibodies or the substrate-specificity of enzymes;
- in contrast to antibodies which can also specifically recognize carbohydrate-residues of glycoconjugates, lectins are of non-immune origin;
- in contrast to enzymes which can also specifically recognize carbohydrates or glycoconjugates, lectins do not display any detectable enzymatic activity;

- 1 - they display carbohydrate-inhibitable homotypic and heterotypic agglutinating activity (see Fig. 1), e.g. of bacteria or blood cells as trypsinized and glutaraldehyde treated rabbit erythrocytes.
- 5 From this definition of lectins it can be taken that for the clear identification of a protein as a lectin, the properties of the protein have to fulfil all the above-mentioned prerequisites. Otherwise the protein in question could, for instance, also be an antibody or an enzyme.

10

The binding of lectins to their corresponding carbohydrates can be either Ca^{2+} -dependent or Ca^{2+} -independent, i.e. some of the lectins only form a complex with the respective carbohydrate in the presence of Ca^{2+} -ions.

15

Until very recently lectins were thought to be peculiarities of the plant kingdom. The physiological role of these proteins is still not known. During the last few years it has become apparent that lectins are regular components of almost every cell membrane or cell surface. Although not much is known yet in this field of research, it is suggested that lectins play a key role in many intercellular processes together with the corresponding carbohydrates on other cells. They form what is probably the most important cellular recognition and communication system and might be important in the development of organs, especially in the development of the central nervous system. Furthermore, they are believed to play a role in fertilization (when sperm and egg recognize each other) and are important in endocytosis; see Barondes, Ann.Rev. Biochem. 50 (1981), p. 207.

Gabius et al. (Hoppe-Seyler's Z. Physiol. Chem. 355 (1984), p. 633), describe Ca^{2+} -independent lectins which were isolated from bovine pancreas and have a molecular weight of 16,000, 35,000 and 64,000, respectively. They bind

1 specifically the β -galactosides lactose and asialofetuin and the
 α -galactoside melibiose. Furthermore, fucose-binding lectins
which are Ca^{2+} -dependent and have a molecular weight of
34,000; 62,000; and 70,000; respectively, are described.

5

Ashwell et al. (Ann. Rev. Biochem. 51 (1982), p. 531)
describe β -galactoside-specific receptors of the liver
which specifically recognize asialo-glycoside residues
of proteins and are responsible for the uptake of these
10 glycoproteins into hepatocytes. Furthermore, a hepatic
mannan-specific receptor is described.

Kawasaki et al. (J. Biochem. 88 (1980), p. 189) (J. Biochem. 94 (1983), p. 937)
published data of a protein with Ca^{2+} -dependent mannan-binding
specificity. The protein was isolated from the mesenteric
15 lymph nodes of rats and from human serum. It was, however, not analyzed
according to the parameters given above, whether this
protein actually is a lectin.

Rutherford et al. (FEBS Lett. 136 (1981), p. 105) describe
the isolation and characterization of a mannan-binding
20 lectin of the vitelline membrane of the early chick embryo.
The physiological role of this protein, however, is not
disclosed.

From a publication of Roberson and Barondes (J. Biol.
Chem. 257 (1982), p. 7520) a lectin of *Xenopus laevis*
25 oocytes, *X. laevis* embryos and the liver of the adult *X.*
laevis is known. The lectin under investigation displays
different specific activities in the three different
differentiation stages.

Grabel et al. (Cell 17 (1979), p. 477) published the occur-
30 rence of a carbohydrate-binding component on the surface
of teratocarcinoma stem cells. This component is designated
by the authors as a lectin-like component and not as a
lectin. Thus, this reference does not disclose whether
or not the carbohydrate-binding component found is a
35 lectin.

Moreover, this publication does not contain any character-
izing data concerning the carbohydrate-binding component.

1 What is disclosed there is just an observation on the
association of cells, which can be inhibited by the
addition of mannose-rich glycoproteins as yeast invertase,
yeast, mannans and horse radish peroxidase. A further publication of
5 Grabel et al. (Biophys., Biochem. Res. Comm. 102 (1981), p.1165) refers
to the extraction of mouse teratocarcinoma cells. According to the
authors, this extract contains a fucoidan-inhibitable hemagglutination
activity.

In the papers of Raz et al. (Cancer Res. 41 (1981), p.
10 3642), Roche et al. (J. Cell. Biochem. 22 (1983), p.
131) and Teichberg et al. (Proc. Natl. Acad. Sci. USA
72 (1975), p. 1383) a β -galactoside specific hamagglutination
activity, a glucose-specific endocytosis activity, and a β -ga-
lactoside -specific hamagglutination activity, respectively,
15 are described which were detected on the surface of tumor
cells or in the extracts of tumor cells.
It has to be understood, however, that the papers of
Grabel et al. (supra), Raz et al. (supra), Roche et al.
(supra), and Teichberg et al. (supra) do not show the presence
20 of lectins on the surface or in the cytoplasm of tumor
cells. The presence of lectins is only proved if all of
the above-mentioned parameters characterizing a protein
as a lectin are investigated. If such a complete charac-
terization is not carried out, the carbohydrate-specific
25 protein may also be an enzyme of the cellular carbohydrate
and glycoconjugate metabolism, see e.g. Roseman (Chem.
Phys. Lipids 5 (1970), p. 270). In this publication of
Roseman, the occurrence of glycosyl-transferases as cell
surface-exposed carbohydrate-specific proteins has been
30 suggested. This hypothesis was confirmed by e.g. Rauvala
et al. (Proc. Natl. Acad. Sci. USA 80 (1983), p. 3991).

35

Finally, lectins were identified in chicken liver and
embryonic chicken muscle (Ceri et al., J. Biol. Chem. 256
(1981), p. 390; de Waard et al., J. Biol. Chem. 252 (1976),

1 p. 5781), human lung (J.T. Powell, Biochem. J. 187
(1980), p. 123) and human liver (Wild et al., Biochem. J.
210 (1983), p. 167).

5 Thus, lectins of mammalian tumor cells have not been
characterized.

Brief Summary of the Invention

10 One object of the present invention therefore is the provision
of specific carbohydrate-binding proteins (lectins) which are
obtained from a mammalian tumor cell.

A further object of the present invention is the provision
15 of specific carbohydrate-binding proteins (lectins) which
are obtained from a mammalian tumor cell and which are
responsible for specific surface properties of said tumor
cell.

Still further, it is an object of this invention to provide
20 a process for obtaining lectins from mammalian tumor cells
which are responsible for specific surface properties.

In the process of the present invention the tumor tissue
25 is first extracted with acetone, precipitating the protein
and thus separating it from e.g. lipids. The acetone from
the precipitate is then evaporated to obtain an acetone powder. The acetone
powder is extracted with a suitable buffered aqueous
solution in order to solubilize the lectin(s). Then the
30 resulting aqueous extract is subjected to at least one
affinity chromatography using columns to which carbohydrates
which can be recognized by lectins are bound. Typical
examples of such carbohydrates are lactose, asialofetuin,
melibiose, mannan, fucose, invertase and heparin. Lactose
35 and asialofetuin are classified as β -galactosides, melibiose
as an α -galactoside. In this chromatography, lectins which
are specifically recognizing

1

the carbohydrate bound to the column will bind themselves to said columns. Subsequently these lectins are eluted from the column using an aqueous solution of the respective

5 carbohydrate having e.g. a concentration of 0.3 or 0.5 M. Finally, the lectins are investigated with respect to their molecular weight and to their properties in hemagglutination-, enzyme activity- and aggregation-assays.

10 Essentially according to this method lectins of a rat rhabdomyosarcoma, a rat fibroadenoma, a rat invasive tubulopapillary adenocarcinoma with a low degree of differentiation, a rat non-invasive tubulopapillary adenocarcinoma with a high degree of differentiation,
15 a murine teratoma, a human malignant epithelial tumor, a human teratocarcinoma (H12.1), a human embryonic carcinoma (H23), a human yolk sac carcinoma, a rat osteosarcoma and of a human sarcoma (Ewing's sarcoma) were isolated and characterized.

20

The lectins of the present invention can be used to provide corresponding monoclonal antibodies and subfragments thereof. Monoclonal antibodies, e.g. mouse or human antibodies, are isolated from suitable producer cells,
25 e.g. hybridoma cell lines, according to known methods.

Anti-lectin-antibody-subfragments, such as the Fab and $F(ab'_2)$ fragments can be prepared by proteolytic cleavage of the antibody molecule with the enzymes papain
30 and pepsin, respectively, followed by purification.

The monoclonal anti-lectin-antibodies or their subfragments, or lectins, or carbohydrates which are recognizable by said lectins can be conjugated with a
35 chemotherapeutic or biologically active compound (such as 5-fluoruridine, vincristine, daunomycine or methothrexate),

1 with a fluorescent or radioactively labelled group
or with another compound permitting the detection of
said molecules in a suitable assay for differential
diagnosis of tumor types and the developmental stage
5 of tumors.

Finally, the molecules referred to above can be used to
provide diagnostic and pharmaceutical compositions, use-
ful for rapid, reliable and precise clinical diagnosis,
10 for scientific research, and for highly specific tumor
therapy and inhibition of metastasation in mammals and
preferentially in humans. The pharmaceutical compositions
containing at least one type of said carbohydrates are
applicable also in a state of neoplastic disease,
15 where the risk of metastasation is strongly increased,
e.g. after surgical treatment.

The molecules referred to above can be utilized in a
composition such as tablet, capsule, solution or sus-
20 pension. They may be compounded in conventional manner
with a physiologically acceptable vehicle or carrier,
excipient, binder, preservative, stabilizer, flavor,
etc. as called for by accepted pharmaceutical practice.

25 Detailed description of the invention

In a first embodiment of the present invention five tumor
types were investigated biochemically for the presence and
characteristics of endogenous lectins.

30 A rat rhabdomyosarcoma reveals only Ca^{2+} -independent-
lectin-specificities.

A rat fibroadenoma of the mammary gland was also in-
vestigated. It contains a diverse pattern of lectins.

35 The lectin pattern of a spontaneous invasive rat
tubulopapillary adenocarcinoma of the mammary gland

1 is also diverse.

Additionally, a spontaneously occurring non-invasive rat tubulopapillary adenocarcinoma of the mammary gland was analyzed.

5 The tubulopapillary adenocarcinomas differ in their degree of differentiation and malignancy; the first one has a lower degree of differentiation. Since fibroadenoma and tubulopapillary adenocarcinoma of the rat mammary gland are morphologically similar to their counterparts in
10 humans, these studies on the pattern of carbohydrate-binding lectins also have significance for human breast cancer.

Extracts using 0.2 M NaCl (salt) and 2 % Triton[®]-X-100 (detergent) from a murine teratoma contain at least nine
15 different lectins.

Furthermore, according to the present invention the pattern of different endogenous lectins of a human malignant epithelial tumor was investigated.

20

Additionally, according to the present invention three human testicular tumors were analyzed, namely a human teratocarcinoma (H12.1), a human embryonic carcinoma (H23), and a human yolk sac carcinoma.

25

Furthermore, the lectin pattern of two sarcomas was analyzed, namely of a rat osteosarcoma and of a human sarcoma (Ewings's sarcoma).

30 The lectins of the present invention which were isolated from said tumors and which were not known from any normal mammalian tissue are summarized in Table I.

35

Table I
Endogenous tumor-derived lectins of the present invention

Tumor type	Ca ²⁺ -dependent			Ca ²⁺ -independent		
	Lactose	Asialofetuin	Melibiose	Lactose	Asialofetuin	Melibiose
Rat rhabdomyosarcoma	-	-	-	-	-	-
						60-72 60-72
					29 43 45	
Rat fibroadenoma	52	52	29			
	67	67	67			
	130	130	74			
Rat invasive tubulopapillary adenocarcinoma (low degree of differentiation)	32	-	140	22	n.d.	44
	64	-	-	52	n.d.	46
						13 30 42 45 62
Rat non-invasive tubulopapillary adenocarcinoma (high degree of differentiation)	-	-	29	-	-	-
			35			29 31 50 52
Murine teratoma	24	-	32	-	-	-
Human malignant epithelial tumor	70	-	62	-	-	-
		28 43 45	70		64	~100 62
Human teratoma (H12.1)	-	-	31	-	-	68
Human embryonic carcinoma (H23)	-	-	70	-	-	-
		56 66	31	32	-	-
Human yolk sac carcinoma	56	-	29	29	29	29
		56	56	56	56	56
						62
Rat osteosarcoma	64	-	-	-	-	-
Human sarcoma (Ewing's sarcoma)	52	52	-	-	-	-
	56	56	-	-	-	-

The apparent molecular weight is given in thousands; n.d. = not determined

1 From the above results demonstrating that tumor cells
carry specific lectins on their surface, the present
inventors concluded that lectins and carbohydrates
recognizable by lectins which are located on the sur-
5 face of tumor cells play a key role in the communication
between tumor cells as well as between tumor and "normal"
cells and furthermore in the process of tissue specific
metastatisation (homotypic and heterotypic aggregation, see
Fig. 1A and B). This provides an experimental basis for
10 lectin impact on growth control and proliferation. If neo-
plastic cells expose both the lectin and the correspond-
ing carbohydrate and if

endogenous lectins can have growth stimulating effects
15 like ConA or PHA, then the tumor cells could stimulate
themselves autocatalytically to exponential growth via
the lectin-carbohydrate system. This phenomenon may
indeed be observed in tumor colonies in vitro and in vivo.
Of course, only the two together, the glycoconjugate and
20 its lectin make sense in biological function.

From the above conclusions of the inventors it can be
taken that in contrast to conventional tumor markers,
the endogenous lectins are functional tumor markers,
25 which participate in processes of tumor growth and
spread.

Therefore, the lectin pattern of a tumor in principle
can be characteristic for

- the type of tumor as compared to the nontransformed
30 cell type,
- the developmental stage or degree of differentiation of
the particular tumor,
- the tissue environment of the particular tumor.

Thus, the results of the experiments of the present
35 invention permit the conception of new compositions use-
ful for diagnosis and therapy of said tumors and also

1 of functional tests to detect tumor cells at early stages
of malignancy.

The diagnostic compositions of the present invention
5 are based on the principle that either the tumor cell
specific lectin(s) is (are) detected by the corres-
ponding carbohydrate(s) or by corresponding mono-
clonal antibodies or antibody-subfragments or the tumor
cell specific carbohydrate(s) is (are) detected by the
10 corresponding lectin(s). For the purpose of suitable
assays either the lectin(s), the carbohydrate(s), the
monoclonal antibodies or the antibody-subfragments
are conjugated with a biologically active compound or
a compound permitting the detection of the respective
15 molecules in said assay or alternatively the carbo-
hydrates are radioactively labelled. Examples of use-
ful markers or labels are enzymes, fluorochromes or
spin labels. It is obvious to a person skilled in the
art that immunological assays as RIA (radioimmunoassay)
20 or ELISA (enzyme-linked immunosorbent assay) may be
carried out by using the respective monoclonal anti-
lectin-antibodies or their subfragments.

Advantageously the diagnostic compositions of this in-
25 vention permit a rapid, reliable, and precise analysis
of tumor cells and are therefore inter alia useful for
the determination of malignancy of tumors during surgi-
cal treatments, for the differential diagnosis
for the distinction of tumor types, and for the deter-
30 mination of the developmental stage of a tumor.

As to the pharmaceutical compositions, as yet the
difficult objective in the chemotherapy of cancer and
certain other diseases was the lacking selectiveness
35 of the applied drugs.

1 A main feature of the pharmaceutical compositions of the present invention is the highly specific interaction of lectins and carbohydrates on the one hand and of lectins and monoclonal antibodies or their sub-
5 fragments on the other hand. For better understanding the revolutionary effectiveness of said therapeutic compositions some examples are given in the following.

If chemotherapeutic (e.g. methotrexate) or biologically active substances (e.g. a subunit of the cholera toxin)
10 are conjugated with lectin(s) or with suitable carbohydrate(s) or with monoclonal anti-lectin-antibodies or subfragments thereof (i.e. synthesis of immunotoxins), they can be specifically targeted to tumor cells carrying the specific carbohydrates or the specific
15 lectins. The specific action of the drug on tumor cells and only on these excludes essentially the side reactions of classical chemotherapeutic agents.

Furthermore the metastasation of tumors can be inhibited by oral or intravenous administration of suitable carbohydrate(s) in such amounts that a complex or complexes
20 with their corresponding lectin(s) are formed. Thus, they are blocking the binding site for adhesion between tumor cell and cells of the target organ.

25

30

35

1 Brief Description of the Drawings

Figure 1 shows a model for heterotypic and homotypic cell aggregation via the glycoconjugate-lectin system.

5.

Figure 2 shows the inhibition of homotypic human teratocarcinoma cell aggregation by sugars. The aggregation in the absence of the inhibitor is given in (a), the aggregation in the presence of varying concentrations of L-fucose (o),
10 D-galactose (x) and D-mannose (+) after 15 minutes is given in (b).

The following examples further illustrate the invention.

Example 1

15

Isolation and characterization of lectins derived from mammalian tumor cells

The pattern under investigation includes specificities
20 for α - and β -galactosides, α -mannosyl and α -fucosyl residues. It is divided into categories for dependence of the binding activity on the presence of Ca^{2+} and on the extraction conditions, representing soluble intra- and extracellular proteins and integral membrane
25 proteins.

Typically, acetone powder from frozen and thawed tumor tissue (50 g) was prepared by two successive extractions with 6 volumes of -70°C acetone in a Waring Blendor. The
30 resulting 21 g of powder were extracted twice with 120 ml Buffer A (0.02 M Tris/HCl, pH 7.8, containing 0.2 M NaCl, 1 mM dithiothreitol and 0.01 mM phenylmethanesulfonyl-fluoride). The supernatants were combined and brought to a final concentration of 0.5 % Triton X-100, 25 mM
35 CaCl_2 and 1.25 M NaCl. The residual pellet was extracted

1 twice with 120 ml of Buffer B (0.02 M Tris/HCl, pH 7.8,
containing 0.4 M KCl, 2 % Triton X-100, 1 mM dithio-
threitol and 0.01 mM phenylmethanesulfonyl fluoride),
the extracts were combined and adjusted to a concentration
5. of 25 mM CaCl_2 . Both solutions were separately passed
over a set of five columns (0.9 x 11 cm lactose-, asialo-
fetuin-, melibiose- and mannan-Sepharose 4B, 0.5 x 10 cm
fucose-Sepharose 4B) equilibrated with Buffer C (0.02 M
Tris/HCl, pH 7.8, containing 1.25 M NaCl, 25 mM CaCl_2 ,
10 0.05 % Triton X-100 and 1 mM dithiothreitol). The
column resins (lactose-, asialofetuin-, melibiose-,
mannose- and fucose-Sepharose 4B, using Sepharose 4B
from Pharmacia, Freiburg, FRG and Carbohydrates from
Sigma, Munich, FRG) have been prepared after suitable
15 activation (divinyl sulphone, cyanogen bromide from
Merck, Darmstadt, FRG) according to standard procedures.
Also reductive amination of disaccharides to an amino
ethylated polyacrylamide support is possible. Asialo-
fetuin was prepared from fetuin (Sigma, Munich, FRG) by
20 desialylation at pH 2 and 80°C. After
extensive washing of the columns, elution of the Ca^{2+} -de-
pendent carbohydrate-binding proteins from the salt extract
and the detergent extract was performed using Buffer D
(Buffer C containing 4 mM EDTA instead of 25 mM CaCl_2).
25 The material was pooled, readjusted to 25 mM CaCl_2 and
adsorbed to a smaller column of the corresponding resin
(3 ml volume). As was noted before, it is advantageous
to perform the second elution with the specific sugars
(0.5 M lactose, 0.5 M melibiose, 0.5 M D-mannose, 0.5 M
30 L-fucose). In general, elution with a molarity of 0.3 has
proved sufficient for complete elution of lectins. The
 Ca^{2+} -independent carbohydrate-binding pro-
teins were eluted by application of Buffer C + 0.5 M
of the specific sugar from the first set of columns that
35 had been reequilibrated with Buffer C. The sugar was re-
moved by dialysis and affinity chromatography was re-
peated on small columns (3 ml).

1 Specificity at this stage was checked by unsuccessful
binding of lectins in the presence of specific sugars
(0.3M) and by in vitro binding to cytochemical
markers on nitrocellulose (Gabijs et al., J. Natl. Cancer
5 Inst. 73 (1984), p. 1349). Furthermore, bound lectins
were not elutable by unspecific sugars like sucrose.

This procedure allowed the separation of Ca^{2+} -dependent
lectins from Ca^{2+} -independent lectins. In this example
10 five carbohydrate specificities were tested. Of course,
many other specificities can be tested in the same way,
such as specificities for neuraminic acid, rhamnose,
heparin, galactosamine, glucosamine and methylated and
acetylated derivatives of these carbohydrates.

15 The samples were concentrated by ultrafiltration using a
membrane as filter (YM5 filter, Amicon). All lectins were
characterized with respect to homogeneity and molecular
weight by polyacrylamide gel electrophoresis in the
20 presence of 0.1 % sodium dodecyl sulfate on 20 x 20 cm
10 % running gels with a 3 % stacking gel. The gels were
stained with Coomassie blue for heparin-inhibitable
lectin or by the silver staining method according to
Morrissey (Anal. Biochem. 117 (1981), p. 307) for all
25 other samples.

Standards for molecular weight designation were: phosphory-
lase b (97 kDa), bovine serum albumin (66 kDa), egg
albumin (44 kDa), glyceraldehyde-3-phosphate dehydrogenase
(36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa),
30 β -lactoglobulin (18.4 kDa).

Furthermore the lectins were optionally characterized by
hemagglutination, enzyme and aggregation assays. Lectin
activity was assayed in microtiter plates with V-shaped

- 1 bottoms with glutaraldehyde-fixed, trypsin-treated
rabbit erythrocytes that in the case of heparin-inhibitable
lectin were pretreated with ethanol. All
agglutination assays were scored after 1 h at room
5 temperature.

Enzyme assays for β -galactosidase, sialyltransferase
and fucosyltransferase, using asialofetuin as potential
acceptor, α -mannosidase and α -fucosidase were performed.

- 10 The sensitivity of the
assay varies from a detection limit of 1.5×10^{-8} unit
of enzyme activity for transferases to 5×10^{-4} unit
of enzyme for glycosidases (1 unit = μ mol of substrate
converted per min). Aggregation of cerebroside vesicles
15 (12 mol % N-plamitoyl-DL-dihydrolactocerebroside) by
 β -galactoside specific lectins was performed at a con-
centration of 7 μ g/ml lectin.

- With each isolation procedure identical results were
20 achieved at least twice.

Example 2

- Isolation and characterization of lectins derived from a
25 spontaneous rat rhabdomyosarcoma, a rat fibroadenoma, two
rat tubulopapillary adenocarcinomas and a murine tera-
toma

- Basically according to the procedure described in
30 Example 1 five different tumor types were investigated
biochemically for the presence and characteristics of
endogenous carbohydrate-binding proteins (lectins).

- The tumors had developed spontaneously in female rats
35 or mice which were obtained from the breeding colonies
of the Central Institute for Laboratory Animal Breeding,
Lettow-Vorbeck-Allee 57, 3000 Hannover 91, West Germany.

- 1 The rat rhabdomyosarcoma originated from the thoracic
cavity of an inbred Brown Norway rat (BN/Han) attached to
the cranioventral section of the sternum and the ribs. The
second tumor, a fibroadenoma of the mammary gland, was
5 found in a 9 months old femal breeder rat of the Han:
SPRD outbred stock which was removed from the breeding
colony of the institute for routine hygienic monitoring.
The third and the fourth tumor can both be classified as
belonging to the tubulopapillary adenocarcinoma group.
10 The third tumor, an invasive tubulopapillary adenocar-
cinoma, had developed in the inguinal area of an outbred
Sprague-Dawley rat (Han:SPRD) and was observed in a life-
span study maintaining rats from weaning up to their
natural deaths. The fourth tumor, an non-invasive rat
15 tubulopapillary adenocarcinoma displays a higher degree
of differentiation than the third tumor and was obtained
from an inbred BDII/Han rat.

- The murine teratoma developed in a 6 months old Han:NMRI
20 mouse in the left ovary. This mouse teratoma was well
differentiated, consisting of various tissues as bone,
cartilage, connective tissue, striated and
smooth muscle cells, nervous tissue including retina,
and an epithelial component.

25

- The purification and characterization of lectins started
from 6 g of a rat rhabdomyosarcoma, 22.7 g of a rat
fibroadenoma, 27 g of an invasive rat tubulopapillary
30 adenocarcinoma, 15.5 g of a non-invasive tubulopapillary
adenocarcinoma and 28 g of a murine teratoma.

35

rat
1 The rhabdomyosarcoma was homogenized in 6 volumes of
extraction medium (75 mM $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.2,
75 mM NaCl, 4 mM β -mercaptoethanol, 2 mM EDTA and
5 0.01 mM phenylmethanesulfonyl fluoride (MEPBS) con-
taining 1 M NaCl, 0.2 M lactose and 0.2 M mannose).
After centrifugation and dialysis first against MEPBS,
later against a buffer with Tris-HCl (75 mM) instead of phos-
phate, raised successively from pH 7.5 to pH 7.8, the
solution was adjusted to 20 mM CaCl_2 and successively
10 passed over a set of columns (0.9 x 12 cm) equilibrated
with buffer A (75 mM Tris-HCl, pH 7.8, 25 mM CaCl_2 ,
4 mM β -mercaptoethanol, 2 mM EDTA, 0.01 mM phenylmethane-
sulfonyl fluoride and 1 M NaCl). The columns were pro-
cessed as in Example 1. The extract, after passing over
15 the columns, was concentrated, submitted to a column
chromatography with Sepharose CL-2B (Pharmacia, Freiburg,
FRG) and dialyzed in the presence of 40 ml heparin-
Sepharose 4B against 0.01 M Tris-HCl, pH 8.6, 4mM
 β -mercaptoethanol and 0.3 M NaCl. Elution from the columns
20 was performed by two means:

- a) with buffer A after omission of CaCl_2 and addition of
4 mM EDTA,
- b) after reequilibration with buffer A using buffer A +
25 0.5 M of the appropriate sugar (lactose, melibiose,
mannose, fucose).

After dialysis of the samples against buffer A, the
affinity chromatography for analysing the lectin pattern was
30 repeated using columns with a capacity of 5 ml.

The lectin pattern of the other tumors was analysed exactly
as described in Example 1.

35 Furthermore, the lectins isolated from said rat and mouse
tumors were subjected to characterization by gel electro-
phoresis, hemagglutination

1 assays and enzymatic assays as described in Example 1.

The lectins which were obtained and which were not known
from any type of normal mammalian tissue are given in Table I.

5

Example 3

Isolation and characterization of lectins derived
from a human malignant epithelial tumor

10

The tumor was surgically removed from the left flexura
of the colon of a 60 year old woman.

The preparation of the lectins from the tumor tissue
was carried out as described in Example 1 starting with
15 34 g frozen and thawed tumor material.

The preparation was carried out three times with identical
results.

20 Subsequently the lectins of this epithelial tumor were
further characterized by demonstrating hemagglutinating
activity and excluding any detectable enzymatic activity
according to the methods given in Example 1.

The lectins obtained from this human epithelial tumor
25 which were not known from any type of normal mammalian
tissue are summarized in Table I.

Example 4

30 Isolation and characterization of lectins dervied from
different human testicular tumors

Essentially according to the procedure given in Example 1
the lectin pattern of a human teratocarcinoma was analyzed.

35

- 1 The teratocarcinoma cell line H12.1 had been established from a primary human testis tumor and was subcultured more than 60 times in vitro. The culture was maintained in RPMI 1640 medium (Flow Labs, Meckenheim, FRG) containing 15 % heat inactivated fetal calf serum (Biochrom, Berlin, FRG), 10 % tryptose phosphate broth (Flow Labs, Meckenheim, FRG), 2 mM L-glutamin, 100 I.U./ml penicillin and 100 µg/ml streptomycin. For transplantation athymic nude NMRI mice (nu/nu, 6 - 8 weeks old) were treated subcutaneously with approximately 10^7 cells. After 2 months the tumors were removed, immediately frozen in liquid nitrogen and stored at -80°C . For the preparation of lectin from the culture, cells were washed with buffer (75 mM Tris/HCl, pH 7.8, containing 1 mM phenylmethanesulfonyl fluoride, 2 mM dithiothreitol and 1 mM NaN_3), scraped out and frozen. Homogenization of 1 g cells (wet weight) was carried out with the same buffer containing 2 % Triton X-100 and lacking NaN_3 .
- 20 In a typical preparation of lectins, acetone powder of tumor material (14 g) was extracted and fractionated as given in Example 1.

All samples after two cycles of affinity chromatography were concentrated by ultrafiltration using a membrane as filter (Diaflo Ultrafiltration Model 50 with a YM-5 membrane). Detergent was removed by chloroform extraction and the heparin-specific lectin was isolated from the tumor material. Subsequently, the lectin pattern was analysed according to example 1, including tests for hemagglutinating and enzyme activity.

To demonstrate the possible functional role of the lectins of said human teratocarcinoma cells, the binding of erythrocytes to these teratocarcinoma cells was monitored in a simple visual assay using trypsinized, glutaraldehyd-fixed rabbit erythrocytes (rosette formation). Since carbohydrate

1 structures on the surface of erythrocytes apparently are
 recognized by carbohydrate-binding proteins of the terato-
 carcinoma cells during the heterotypic recognition, inhi-
 5 bition of this process by addition of sugars and glyco-
 proteins was tested (Table II).

Table II
 Inhibition of rosette formation

10	Inhibitor	% Inhibition of rosette formation
	N-acetyl-D-galactosamine	0
	L-fucose	2
	D-galactose	4
15	N-acetyl-D-glucosamine	0
	D-mannose	10
	fetuin	0
	asialofetuin	7
	asialo-agalactofetuin	0
20	mannan	21
	Invertase	34
	Invertase (periodate-oxidized)	4
	lactose-BSA	7
	mannose-BSA	14
25		

Saccharides were added at 0.2 M, glycoproteins at 1 mg/ml. All re-
 sults are averages from 8 - 10 independent experiments.

Whereas monosaccharids as D-mannose and D-galactose only
 30 slightly inhibited the heterotypic aggregation at 0.2 M
 concentration, a more pronounced effect was seen with
 glycosylated bovine serum albumin (lac-BSA, man-BSA).
 Since galactose-binding proteins were known to bind to
 the mannose-glycoprotein invertase, the
 35 difference in inhibitory efficiency of invertase in re-
 lation to mannan may indicate a binding of invertase
 to galactose- and mannose-specific sites on the terato-

1 carcinoma cells. No inhibition was seen with N-acetyl-
glucosamine, asialoagalactofetuin, fetuin, glucose and
sucrose. This excludes an unspecific sugar effect on
rosette formation. Bovine serum albumin (BSA) also had
5 no inhibitory influence. Coupling of p-aminophenyl-gluco-
side by diazotation to BSA, as similarly used for the
derivatives of β -lactose and α -D-mannose, does not in-
fluence the inertness of BSA in rosette formation,
excluding any unspecific effect due to the chemical
10 modification procedure. Since the inhibition by inver-
tase is drastically reduced after extensive oxidation
of sugar moieties in invertase by periodate treatment,
the importance of sugars in the recognitive process
during rosette formation is further emphasized.

15 Inhibition of rosette formation carried out as described
above demonstrated the participation of a protein
carbohydrate interaction in heterotypic aggregation.
Since type and abundance of carbohydrate structures
20 of glycoconjugates on erythrocytes and teratocarcinoma
cells might differ significantly, the influence of
sugars on reaggregation of teratocarcinoma cells that
were carefully mechanically dissociated in calcium-
and magnesiumfree phosphate-buffered saline was
25 tested. The tests revealed a similar inhibition
pattern for the homotypic aggregation in relation to
the heterotypic system with mannose and galactose being
effective inhibitors (Fig. 2). The ability of D-mannose
and D-galactose to inhibit aggregation of teratocarcinoma
30 cells further suggested that these sugars interact with
cell surface carbohydrate-binding proteins. In order to
establish the capacity of sugars to bind to carbo-
hydrate-binding proteins of the cell surface, fluorescent,
glycosylated markers provided a versatile cytological tool
35 for visualization. Observation of teratocarcinoma cells
after labelling with markers specific for D-mannose and

1 lactose resulted in significant binding of markers.
The binding was performed at 4°C in order to minimize
internalization or shedding of membrane-bound proteins.
Complete inhibition of binding occurred in the presence
5 of the appropriate sugar (D-mannose or lactose) in a 0.25M
concentration or of 1 mg/ml unlabelled glycosylated BSA.
These data raised evidence that the lectins contained in
the detergent extract of human teratocarcinoma cells
being specific for D-mannose and D-galactose are involved
10 in Ca^{2+} -independent cell-cell recognition.

The human embryonic carcinoma cell line H 23 had been
established from a primary human testicular tumor of
a 26 year old patient and was subcultured more than 30
15 times in vitro. For transplantation, athymic nude mice
(nu/nu, 6 - 8 weeks old) (Central Institute for
Animal Breeding, supra) were injected subcutaneously
with approximately 10^7 cells. After two months the
tumors, histologically determined as embryonic carcinoma,
20 were removed, immediately frozen in liquid nitrogen
and stored at -80°C.

The yolk sac tumor material was obtained by autopsy of a
20 year old boy suffering from a testicular embryonic
25 carcinoma. Viable parts of the abdominal tumor mass were
removed, consisting histologically predominantly of
yolk sac tumor material.

The lectins of said human embryonic carcinoma cell line
30 H 23 and of said yolk sac tumor were isolated and charac-
terized according to Example 1, including functional tests
for hemagglutinating and enzyme activity.

The lectins identified in the above-mentioned human
35 testicular tumors which were not known from any type of
normal mammalian tissue are summarized in Table I.

- 1 The analyzed lectin patterns demonstrate that lectins can
be considered as functional tumor markers useful for
differential diagnosis of tumors and different develop-
mental stages of tumors.

5

Example 5

- 10 Isolation and characterization of lectins derived from
a rat osteosarcoma and a human sarcoma (Ewing's sarcoma)

The lectin pattern of a rat osteosarcoma was determined by
analysis of 3 g tumor tissue as described in Example 1.

- 15 Tumor material of the human sarcoma (Ewing's sarcoma)
was obtained from the Cancer Center of the University
of California, San Diego. 3.5 g of the tissue were
extracted and analyzed for their lectin
pattern as described in Example 1.

20

Subsequently, functional tests for hemagglutinating and
enzyme activity were carried out according to Example 1.

- 25 The lectins obtained from these tumors which were not
known from any type of normal mammalian tissue are
summarized in Table I.

30

35

C l a i m s

1. Specific carbohydrate-binding proteins (lectins) obtained from a mammalian tumor cell and responsible for specific properties of said tumor cell.
2. Lectins according to claim 1, characterized in that they are derived from a rat rhabdomyosarcoma, have the following molecular weight (in thousands) and bind specifically to the following carbohydrates:

Ca²⁺-independent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
-	-	29	60-72	60-72
		43		
		45		

3. Lectins according to claim 1, characterized in that they are derived from a rat fibroadenoma, have the following molecular weight (in thousands) and bind specifically to the following carbohydrates:

Ca²⁺-dependent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
52	52	52		29
67	67	67	52	67
130	130	74	67	

4. Lectins according to claim 1, characterized in that they are derived from a rat invasive tubulopapillary adenocarcinoma with a low degree of differentiation, have the following molecular weight (in thousands) and bind specifically to the following carbohydrates:

Ca²⁺-dependent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
32				
64	-	-	-	140

26

Ca²⁺-independent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
22	-	-	44	13
52			46	30
				42
				45
				62

5. Lectins according to claim 1, characterized in that they are derived from a rat non-invasive tubulopapillary adenocarcinoma with a high degree of differentiation, have the following molecular weight (in thousands) and bind specifically to the following carbohydrates:

Ca²⁺-dependent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
-	-	-		29
			-	35

Ca²⁺-independent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
-	-	29	-	29
		50		31
		52		50
				52

6. Lectins according to claim 1, characterized in that they are derived from a murine teratoma, have the following molecular weight (in thousands) and bind specifically to the following carbohydrates:

Ca²⁺-dependent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
24	-	-	32	32

Ca²⁺-independent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
-	-	-	-	~100

7. Lectins according to claim 1, characterized in that they are derived from a human malignant epithelial tumor, have the following molecular weight (in thousands) and bind specifically to the following carbohydrates:

27

Ca²⁺-dependent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
70	-	28	-	62
		43		70
		45		

Ca²⁺-independent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
-	-	64	-	62

8. Lectins according to claim 1, characterized in that they are derived from a human teratocarcinoma, have the following molecular weight (in thousands) and bind specifically to the following carbohydrates:

Ca²⁺-dependent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
-	-	31	-	31
				70

Ca²⁺-independent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
-	-	-	68	-

9. Lectins according to claim 1, characterized in that they are derived from a human embryonic carcinoma, have the following molecular weight (in thousands) and bind specifically to the following carbohydrates:

Ca²⁺-dependent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
-	-	56	66	31
		66		

Ca²⁺-independent

Lactose	Asilofetuin	Melibiose	Mannan	Fucose
32	-	-	-	-

10. Lectins according to claim 1, characterized in that they are derived from a human yolk sac carcinoma, have the following molecular weight (in thousands) and bind specifically to the following carbohydrates:

28

Ca²⁺-dependent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
56	-	56	-	29 56

Ca²⁺-independent

Lactose	Asialofetuin	Melibiose	Mannan	Fucose
29 56	29 56	29 56	-	29 56 62

11. Lectin according to claim 1, characterized in that it is derived from a rat osteosarcoma, has a molecular weight of 64 000 and Ca²⁺-dependent lactose-binding specificity.
12. Lectins according to claim 1, characterized in that they are derived from a human sarcoma (Ewing's sarcoma), have the following molecular weight (in thousands) and bind specifically to the following carbohydrates:

Ca²⁺-dependent

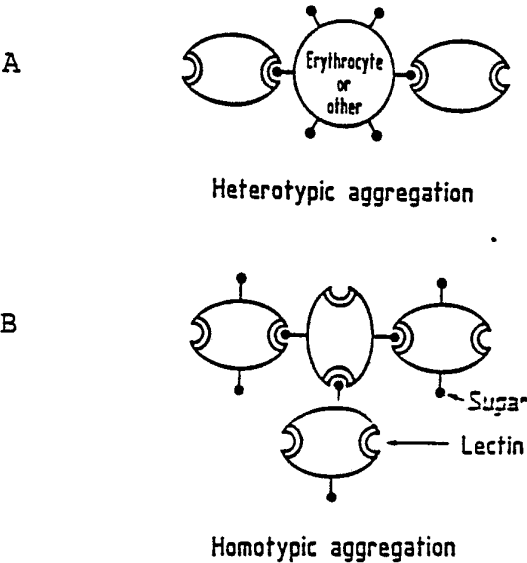
Lactose	Asialofetuin	Melibiose	Mannan	Fucose
52 56	52 56	52 56	-	-

13. Process for obtaining the lectins according to claims 1 to 12 comprising the following steps:
- extraction of the tumor tissue with acetone,
 - evaporation of the acetone extract to obtain an acetone powder,
 - extraction of the acetone powder with a buffered aqueous solution for the solubilization of the lectins,
 - adsorption of the lectins contained in the aqueous extract to affinity chromatography columns to which carbohydrates which can be recognized by lectins are bound,
 - elution of Ca²⁺-dependent lectins from the column with an aqueous solution of a chelating agent, and subsequently
 - elution of Ca²⁺-independent lectins from the column with an aqueous solution of a carbohydrate competing with the carbohydrate bound to the column for the binding site of the lectin.

14. Process according to claim 13, characterized in that the aqueous extract is obtained by homogenisation of the tumor tissue in an aqueous extraction medium and by centrifugation and dialysis of the homogenisate.
15. Monoclonal antibody or a subfragment thereof derived from a human or a murine hybridoma, characterized in that it is specifically directed to a lectin according to any one of claims 1 to 12.
16. Diagnostic composition for the detection of mammalian tumor cells, characterized in that it contains a lectin according to any one of claims 1 to 12, a carbohydrate recognizable by said lectin, a lectin-specific monoclonal antibody or a derivative of said lectin, said carbohydrate or said monoclonal antibody.
17. Method for the detection of mammalian tumor cells, which comprises contacting a lectin according to any one of claims 1 to 12, a carbohydrate recognizable by said lectin, a lectin-specific monoclonal antibody or a derivative of said lectin, said carbohydrate or said monoclonal antibody specifically and in a sufficient quantity with a carbohydrate or a lectin-carrying mammalian tumor cell.
18. Pharmaceutical composition for treating malignant neoplasias by specifically destroying tumor cells and/or inhibiting metastasation containing an effective amount of a lectin according to any one of claims 1 to 12, a carbohydrate recognizable by said lectin, a lectin-specific monoclonal antibody or a derivative of said lectin, said carbohydrate or said monoclonal antibody and a pharmaceutically acceptable carrier or diluent.

19. Method of treatment of malignant neoplasias by specifically destroying tumor cells and/or inhibiting metastasation, which comprises the use of an effective amount of a lectin according to anyone of claims 1 to 12, a carbohydrate recognizable by said lectin, a lectin-specific monoclonal antibody or a derivative of said lectin, said carbohydrate or said monoclonal antibody.

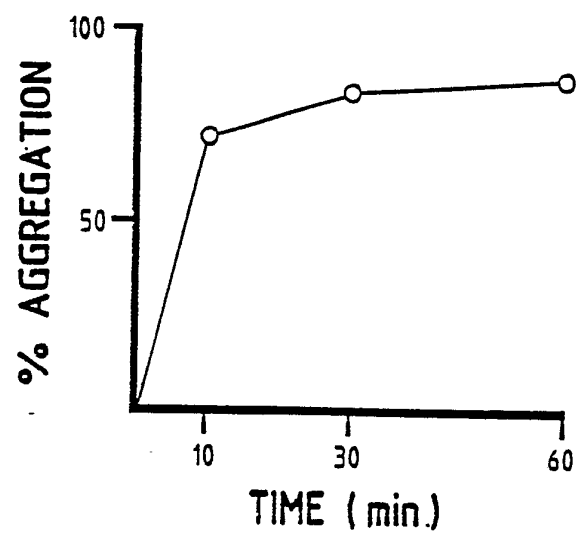
Figure 1



2/2

Figure 2

a)



b)

