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(54) **METHOD FOR DETERMINATION OF THE RESPONSIVENESS OF AN INDIVIDUAL TO MISTLETOE LECTIN**

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(57) **ABSTRACT**

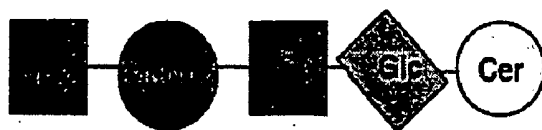
A method for the determination of the responsiveness of an individual to mistletoe lectin or to (an) mistletoe lectin single chain(s), wherein the expression of a membrane-bound receptor for mistletoe lectin is characteristic of a corresponding responsiveness.

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Figure 1

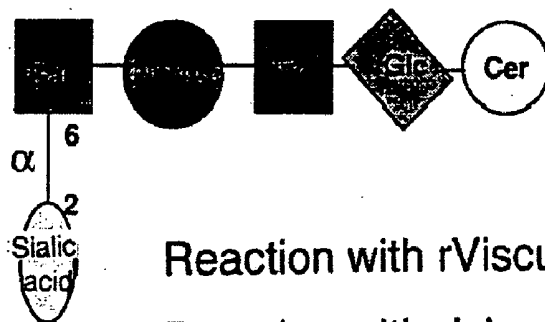
A Neutral glycosphingolipids:



Reaction with rViscumin: -

Reaction with ricin: +++++

B Gangliosides:



Reaction with rViscumin: +++++

Reaction with ricin: -

Figure 2

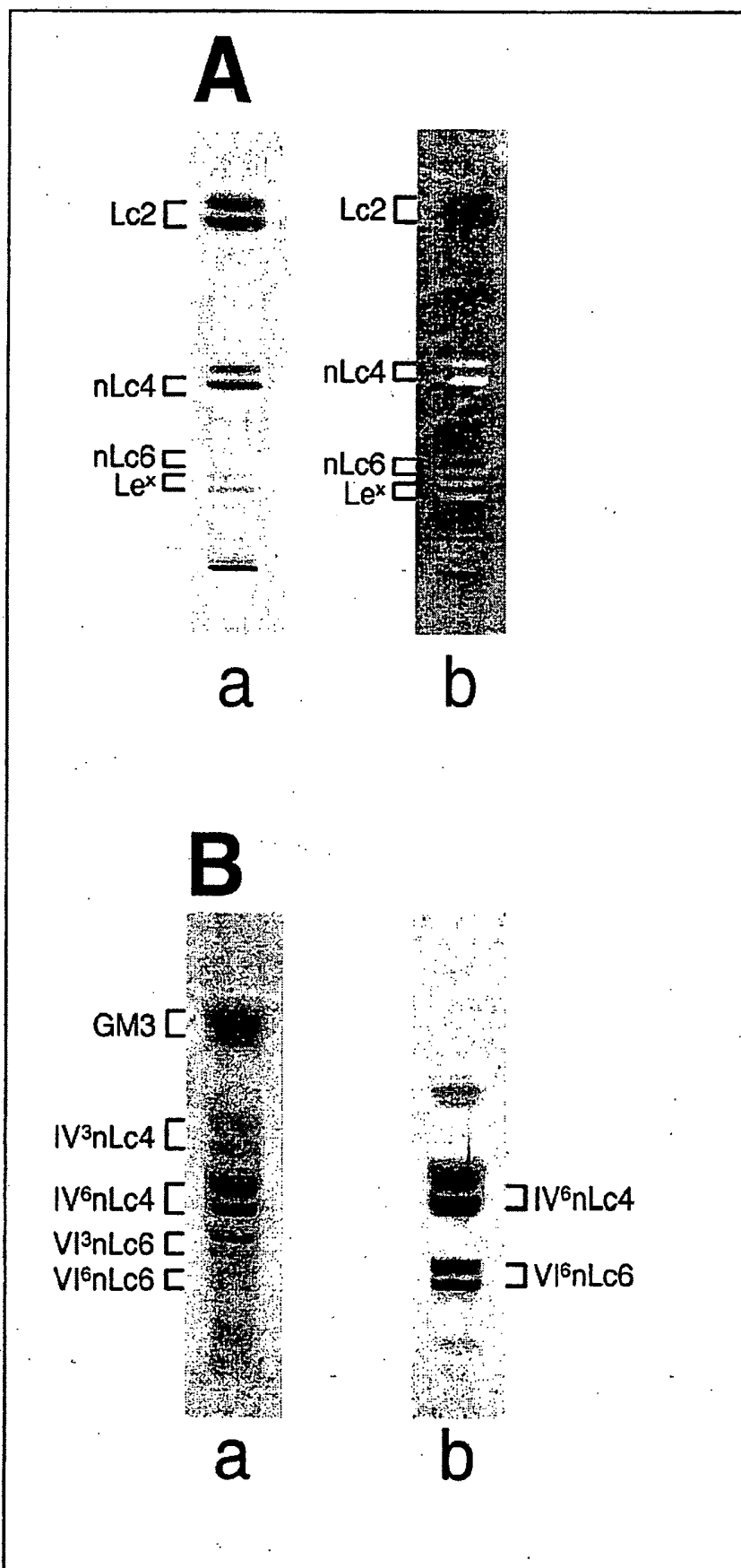


Figure 3

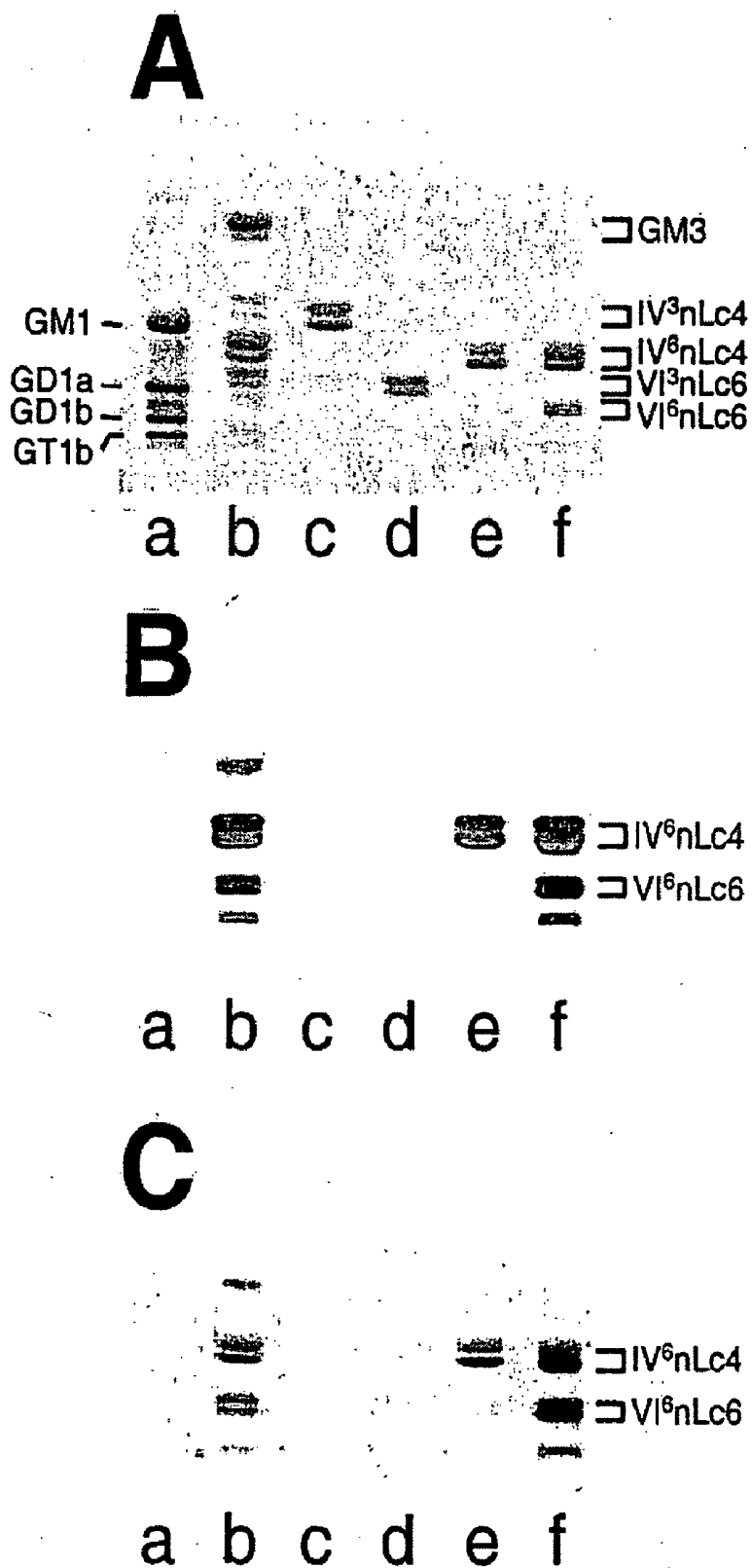


Figure 4

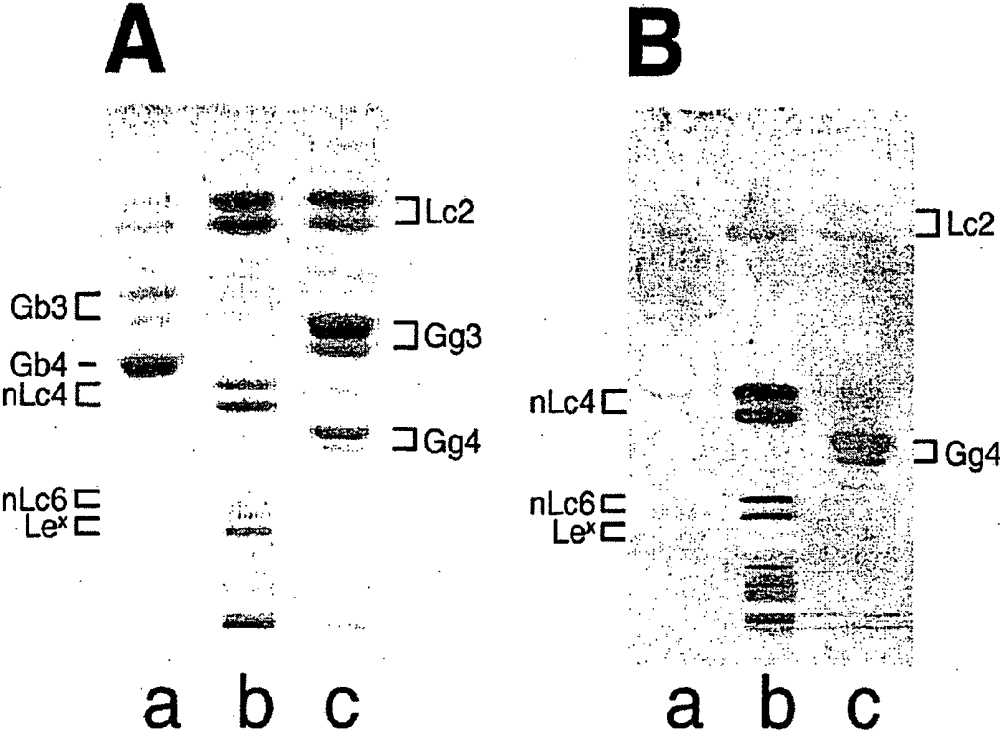


Figure 5

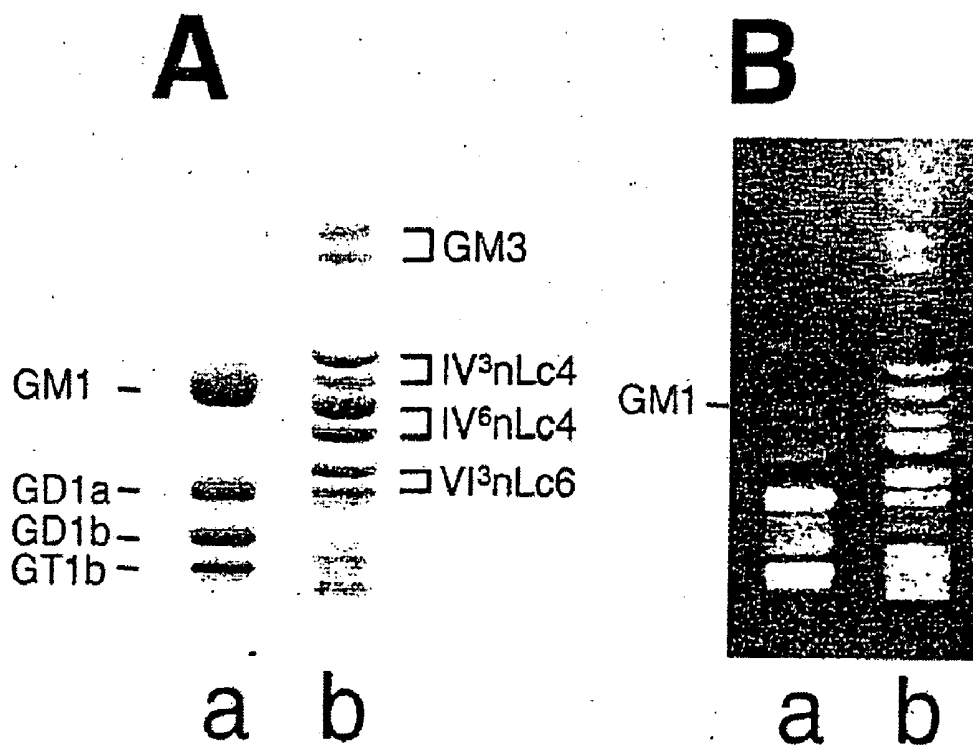


Figure 6

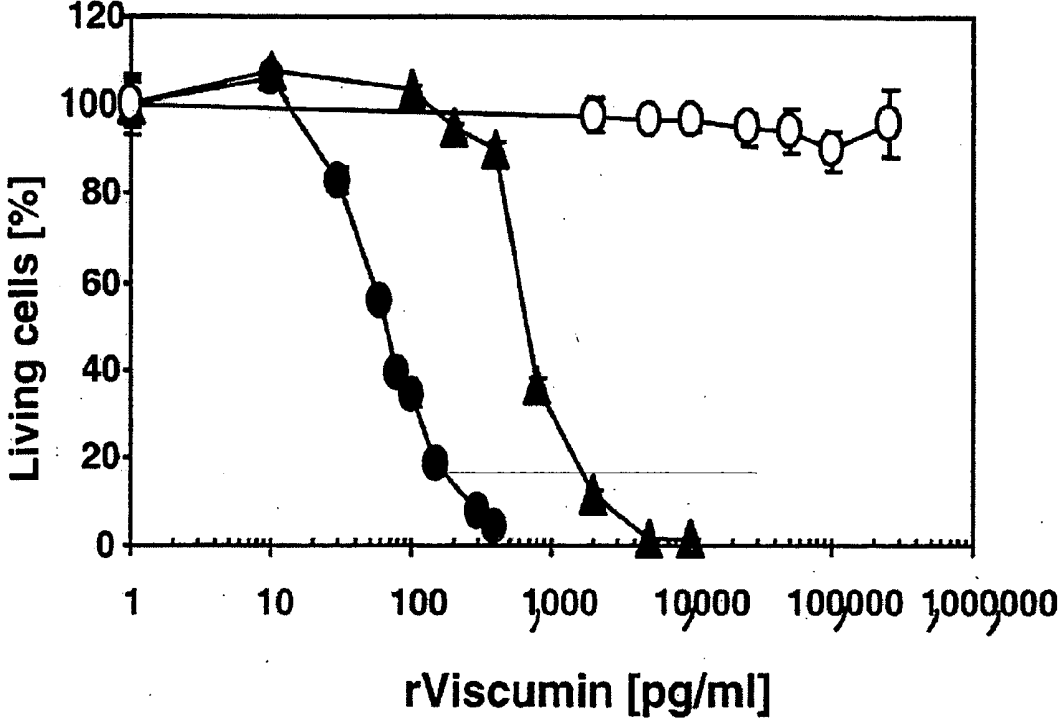


Figure 7

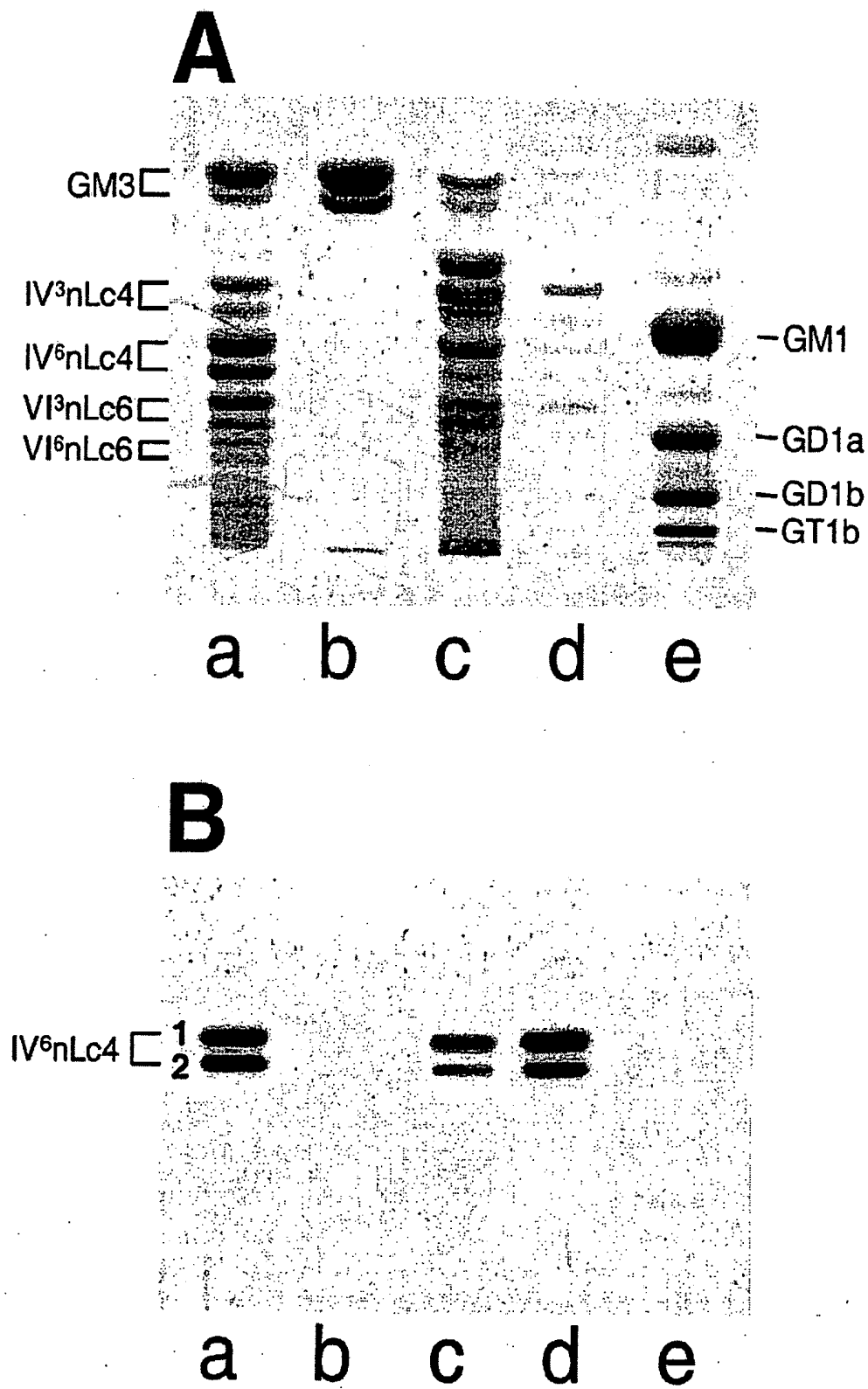


Figure 8

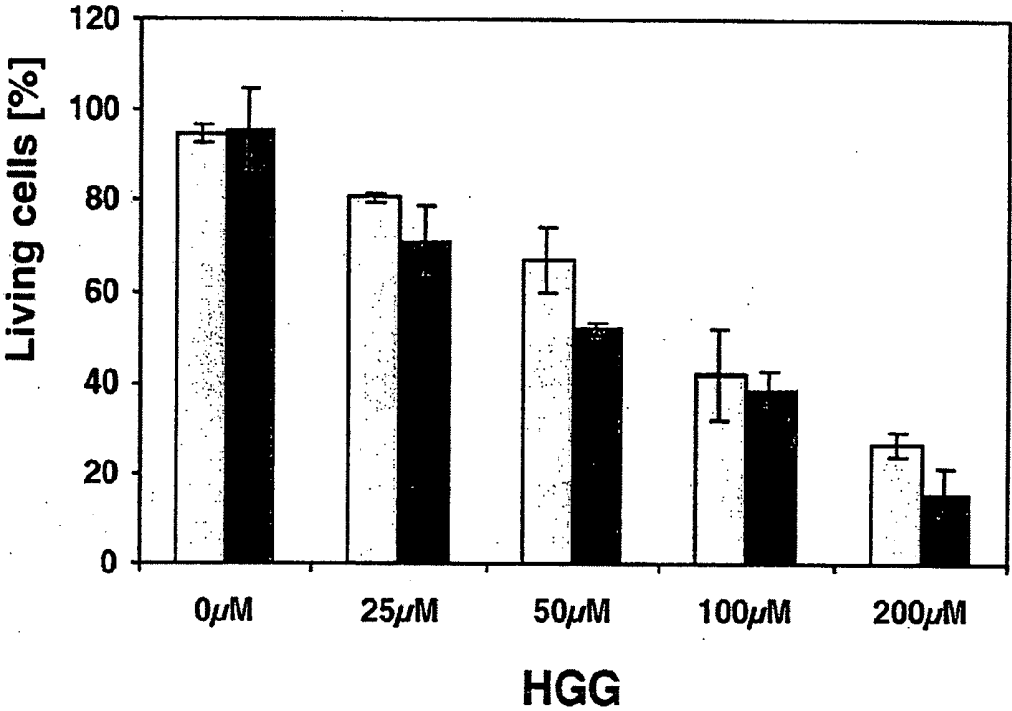


Figure 9

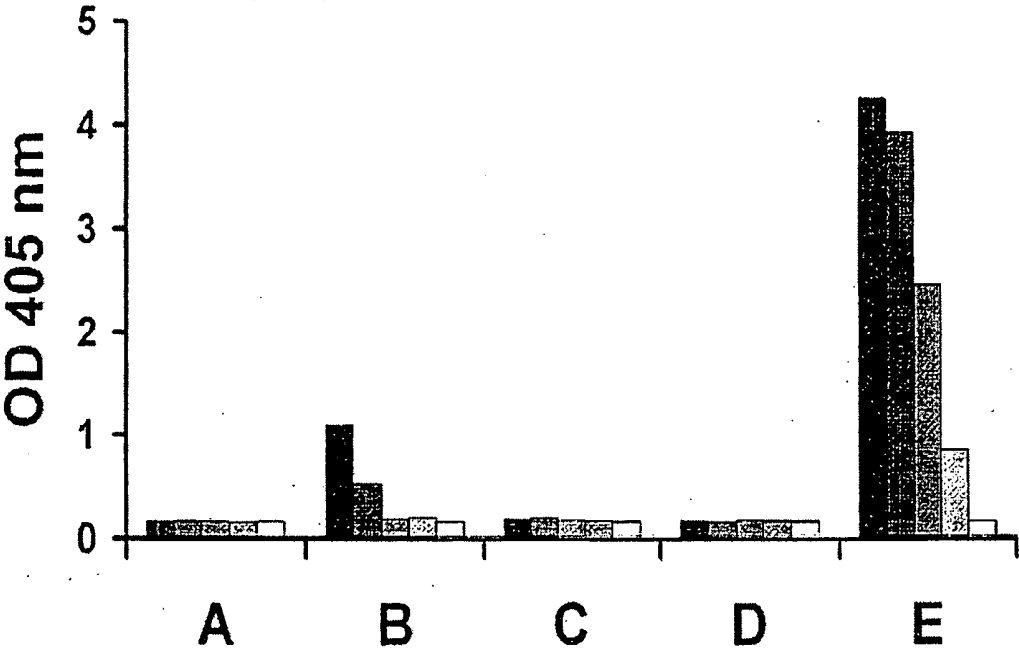
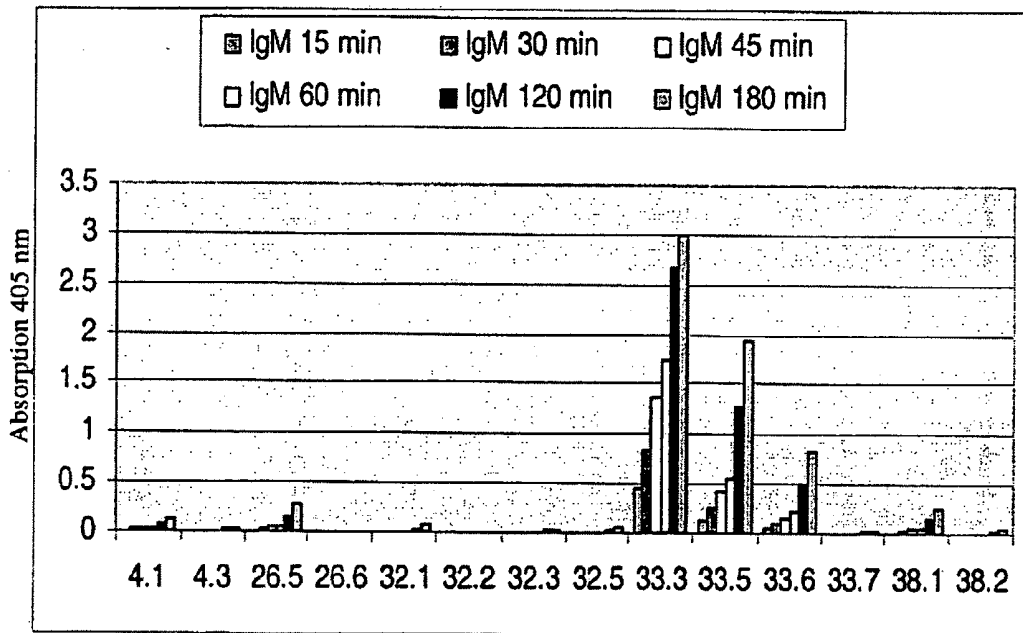


Figure 10A

Test for IgM – AB



Hybridoma No.

Figure 10B

Test for IgM – AB

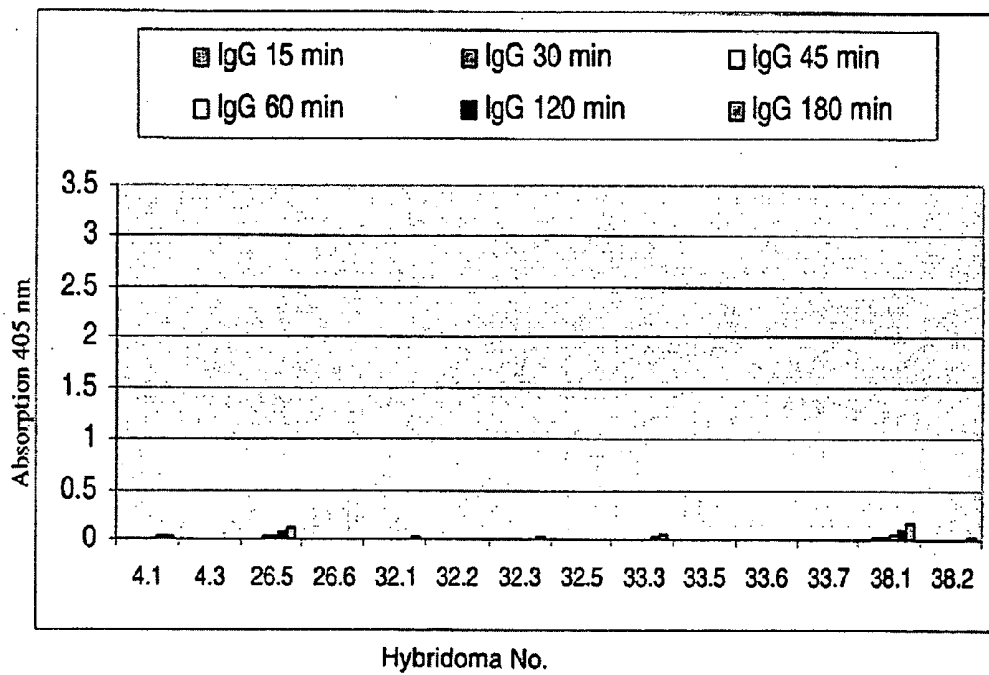


Figure 11

Test for IgM – Production

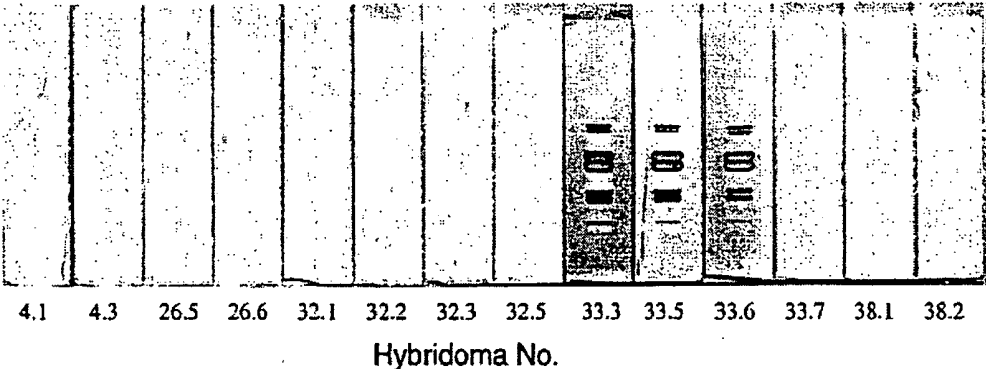


Figure 12

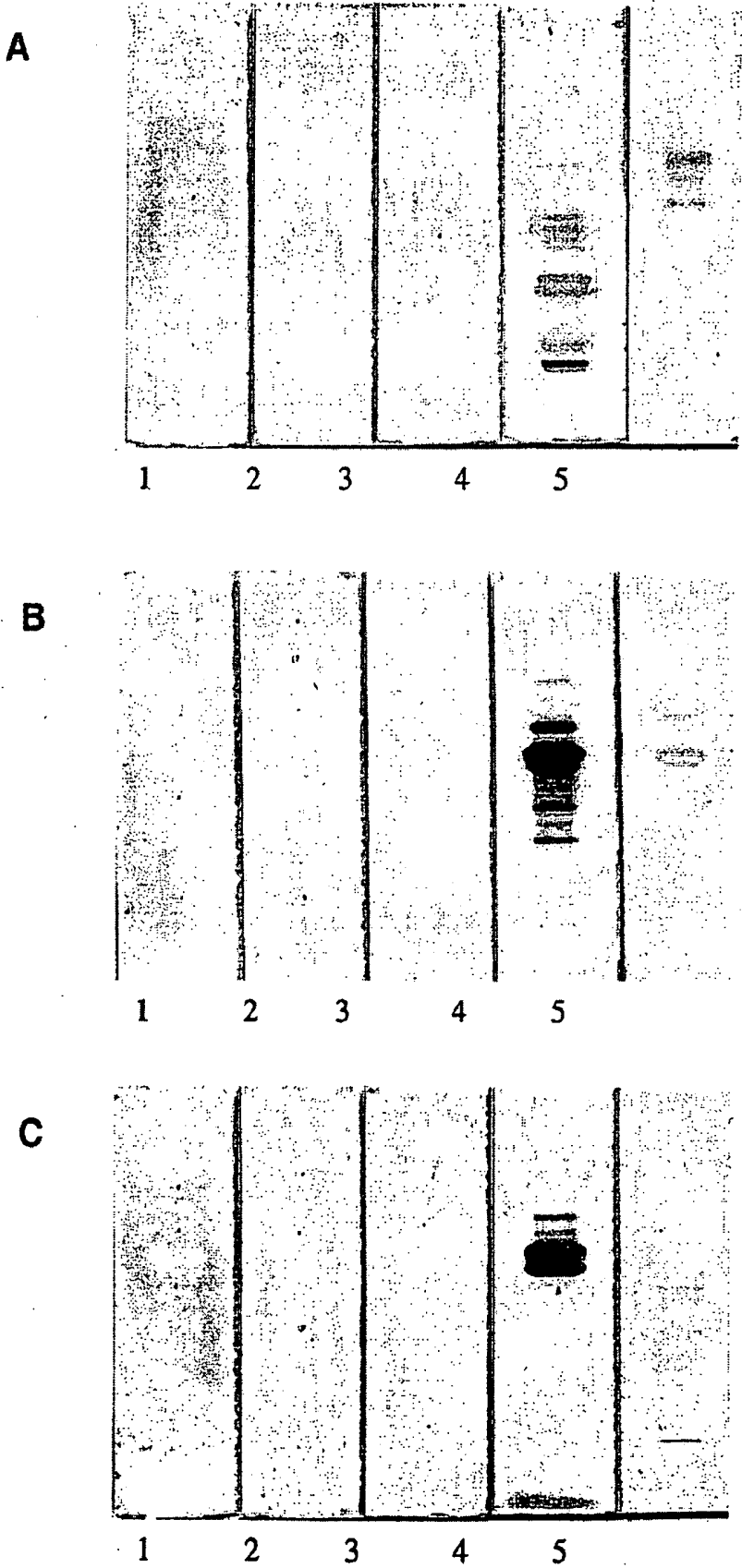


Figure 13

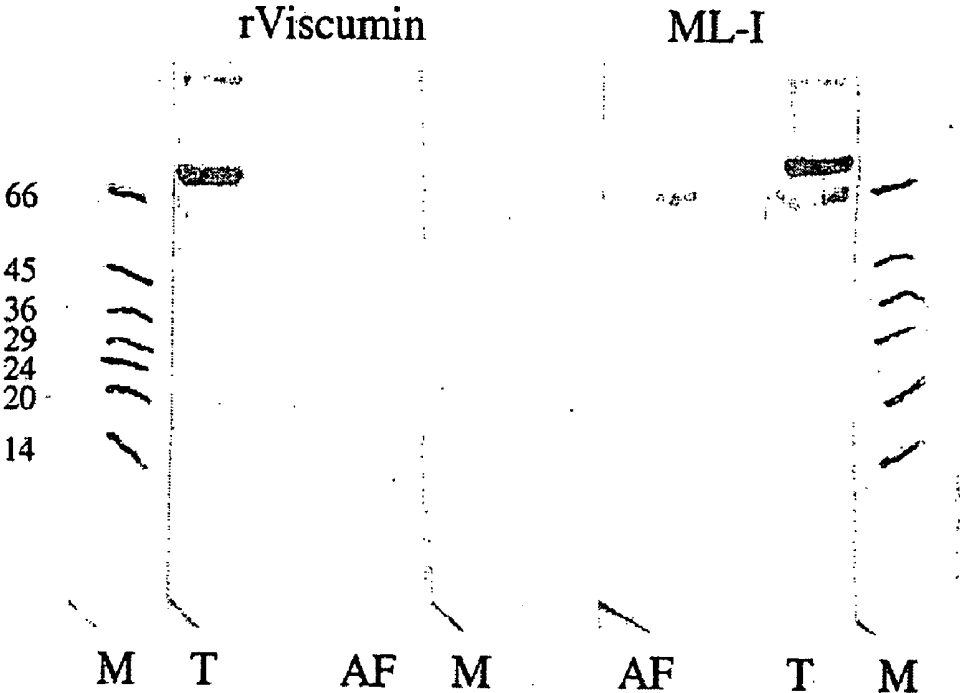
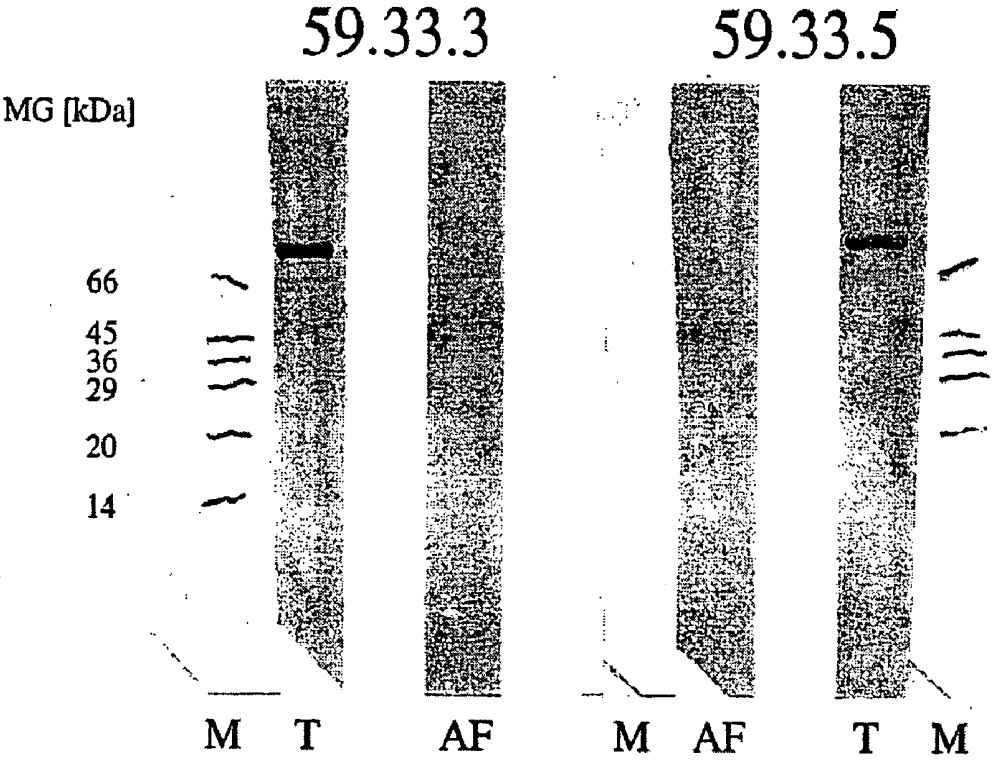


Figure 14



METHOD FOR DETERMINATION OF THE RESPONSIVENESS OF AN INDIVIDUAL TO MISTLETOE LECTIN

[0001] The present invention relates to a method for determination of the responsiveness of an individual to mistletoe lectin or to a mistletoe lectin single chain wherein the expression of a specific, membrane-bound receptor for mistletoe lectin is characteristic of a corresponding responsiveness.

[0002] Various documents are cited in the text of this description. The disclosure content of the cited documents (including all manufacturer's descriptions, instructions, etc.) is herewith incorporated by reference into this description.

[0003] Mistletoe lectin is a type II ribosome-inactivating protein (RIP) related to ricin and formed of two protein chains (Barbieri et al., 1993). In this case, the A-chain has an enzymatic rRNA-N-Glycosidase activity, the B-chain has a carbohydrate-binding activity. Recombinant rViscumin provided in *E. coli* is known to the person skilled in the art (EP 0 751 221 B1). In particular, rViscumin produced in *E. coli* is clinically developed as a mono substance. With regard to its primary structure, rViscumin does not exactly correspond to ML-I, ML-II or ML-III that can be found in the mistletoe plant. It is to be assumed that there are, apart from the rVisum sequence described, further mistletoe lectin variants which are modified by point mutations in the original gene and which can slightly differ in their primary structure. rViscumin could be regarded as a variant of the primary mistletoe lectin and could be understood as a mixture of ML-I, ML-II and ML-III sequences.

[0004] The activities of both chains of the mistletoe lectin are necessary for the cytotoxic/cytostatic activity of the protein. In this case, the first step, i.e. the binding of the molecule on the surface of the cell, is of crucial importance.

[0005] As in the case of ricin, the mistletoe lectin (and the rViscumin recombinantly produced in *E. coli*; Eck et al., 1999a, Eck et al., 1999b), too, is said to have galactose/lactose specificity (Olsnes et al., 1982; Lee et al., 1992; Gilleron et al., 1998). In this case, the kind of glycosidic linking of the terminal galactose and the subsequent sugar have so far been described as not being important for the specificity of the lectin (Lee et al., 1994; Gupta et al., 1996).

[0006] Various mistletoe lectins (ML-I, -II and -III) which differ in their carbohydrate specificity are described in the literature (Franz, 1986). In this case, it is discussed that the specificity of galactose/lactose (ML-I) changes through a mixed form of galactose/lactose and N-acetyl galactosamine (ML-II) to a mistletoe lectin which binds stronger to N-acetyl galactosamine (ML-III). The rViscumin has a galactose/lactose binding activity that is detectable in different ELISA-like methods (Eck et al., 1999b). Other carbohydrate-binding activities of the mistletoe lectins have so far been described very rudimentary only and, in part, in a very contrary manner. Wu et al. (1995a and 1995b); for instance, observed that the capability of ML-I to bind either human α 1-acidic glycoprotein or fetuin is reduced significantly if the desialylated counterparts are used. In contrast thereto, said authors found an increase in the binding and a complete precipitation if sialoglycoprotein from rat in desialylated form was used in the test. However, the conclusions drawn by Wu et al. cannot be interpreted due to the contra-

dictory results of the sialic acid carrying or desialylated proteins used. Furthermore, it is to be noted that the glycosylation of the proteins is not uniform. A desialylation process is a chemical process and varies depending on the manufacturer's indications. In a desialylated sample, for instance, there may still be a residue of proteins carrying sialic acid in a terminal position which falsify the results communicated. Nevertheless, a specificity of ML-I to sialic acid was discussed as the authors succeeded in reducing the protein mistletoe lectin interaction if the oligosaccharide Neu5Ac α 2-3/Neu5Ac α 2-6Gal- β 1-4Glc was used as a competitor. In the tabular summary of the competition results of a precipitation experiment (microprecipitation technique) it is however striking that, if the same test set up was used, a precipitation of ML-I by various glycoproteins (human α -1 acidic glycoprotein; fetuin, asialo RSL) can be prevented by both lactose and the mixture Neu5Ac α 2-3/Neu5Ac α 2-6Gal- β 1-4Glc and Gal β 1-4GlcNAc in about the same concentrations. A specificity of ML-I for sialic acid cannot be clearly seen from these data, also because the results which were obtained with asialo RSL and fetuin carrying sialic acid in a terminal position have the tendency to be absolutely identical.

[0007] At about the same time, Debray et al. (1994) was able to show that the affinity of ML-I immobilised on sepharose 4B to either O-3 or O-6 sialylated lactose or sialo-N-glycosyl peptides has slightly increased compared to N-acetyl-lactosamine-type oligosaccharides and glycopeptides. In this case, Debray et al. (1994) used oligosaccharides and glycopeptides which they partly isolated from human sources (e.g. urine, human serum transferrin, human α -1 acidic glycoprotein). Debray et al. carried out examinations on a column to which they immobilised mistletoe lectin I (ML-I sepharose). When assessing the saccharides tested, they differentiated between three factors. Fraction 1 (FNR) showed no interaction with the mistletoe lectin and eluted in PBS in the elution volume of the column. A second fraction (FR) eluted with slight retardation, however still with use of PBS. Accordingly, saccharides of these two fractions differed only slightly with regard to their capability to interact with the lectin immobilised on the column. The actually binding fraction (FE) could only be eluted by 150 mM galactose in PBS buffer. The results are not conclusive. The authors discussed, for example, a retardation of the elution of FNR compared to FR if they linked a sialic acid α 2-6 to the terminal galactose residues (cf. saccharides 16 (FR) and 17 (FNR)). However, a double sialic acid labelling, as can be seen in saccharide 15 (FR), did not result in the substance binding more strongly to the ML-I on the column. It is however difficult for the skilled person to discuss explicitly the retardation of FNR compared to FR. Thus, the slight retardation (FR) is likely to be caused by unspecific interactions (e.g. hydrophobic interactions) with the protein bound to the column and is not due to the specificity of the mistletoe lectin. Only two of the structures tested eluted after rinsing the column with PBS buffer+150 mM NaCl+150 mM galactose (FE). These two structures were isolated from bovine thyroglobulin or turtledove ovomucoid. In contrast to all other structures which the authors used and mainly contained the structure Gal(β 1-4)GlcNAc-, these structures terminally have two galactose residues which are linked either α 1-4 or α 1-3 to the second galactose.

[0008] Lee et al. (1994) described the relevance of the second sugar residue for the recognition of ML-I. They

divided the recognition of sugar structures by ML-I up into four groups and were able to show that GlcNAc structures at the second position greatly impaired the recognition of the sugars. They found, the strongest affinity of ML-I to a saccharide with the terminal sugar residues β -D-Gal-(1-2)- β -D-Gal-. The β 1-3-linked galactoses, too, showed similarly good specificities. Lee et al. (1994) did not test β 4-linked galactoses, it can however be concluded from the work of Debray et al. (1994) that these, too, should have a high specificity to ML-I.

[0009] When assessing the work of Debray et al. (1994), it must however be noted that the examinations were carried out with mistletoe lectin which had been immobilised on a column. The interactions of the saccharides, which were observed, with the immobilised lectin are an unphysiological experimental system. Thus, it is not possible to directly draw a conclusion with regard to the interaction of dissolved mistletoe lectin or rViscumin in solution with a receptor.

[0010] The data presented herein by Lee et al. (1994), Debray et al. (1994) and Wu et al. (1995) with regard to the specificity of mistletoe lectin contradict each other and cannot be considered a conclusive evidence of a specificity of the mistletoe lectin to Neu5Ac. It is particularly problematic that the data were not carried under defined test conditions. In particular, this refers to the quality of the proteins used which, for technical reasons, never have a homogeneous carbohydrate structure.

[0011] On the basis of the observations by Lee et al. (1992), Galanina et al. (1997) designed an experimental system in which mistletoe lectin was coupled to structurally defined neoglyco-conjugates. In this system, the competitive potency of synthetic oligosaccharides to reverse the binding of mistletoe lectin was examined. Yet, the results obtained with this system are also heterogenous. A competitive potency of lactose was shown as expected. The competition with N-acetyl-lactosamine leads to similar results. Moreover, naturally occurring isomers of the sialyl-lactose were examined, too. In this case, however, the α 2-3 sialylated isomer had a higher competitive activity than the α 2-6 sialylated isomer, which, however, was clearly below the one of lactose or N-acetyl-lactosamine. Moreover, the authors stated that N-acetyl-neuramic acid alone did not have an inhibitory activity. In the analysis of the works based on competitive studies, it is striking that the naturally occurring glycoproteins examined comprised soluble proteins only. Membrane-bound glycoproteins were not examined in the experiments described.

[0012] Mistletoe lectin, but also, for example, other lactose/galactose-specific proteins such as ricin or galectin are described as asialo-fetuin-binding proteins. This property can also be utilised for a quantification (Vang et al., 1986). Gupta et al. (1996) were able to describe the interaction of the proteins ricin, galectin or mistletoe lectin (here designated as *Viscum album* agglutinin) in more detail. They found that all three proteins form defined complexes with asialo-fetuin. The receptor(s) that is (are) responsible for the binding of the mistletoe lectin or ricin to the target cell is (are) not yet known.

[0013] In 1982, the receptor for the cholera toxin was identified on Balb/c 3T3 cells (Critchley et al., 1982). It is the ganglioside GM1. Based on this work, it was tried to search for the receptors of other toxins/lectins in this area,

too. In this case, for ricin and peanut agglutinin it could be shown that the receptors on human lymphocytes are glycoproteins whereas *Ricinus* agglutinin and, soybean agglutinin bind to glycoproteins and gangliosides to about the same degree (Turpin et al., 1984). In examinations with model membranes in which the monosialo ganglioside GM1 (with terminal galactose) was inserted only very unspecific interactions with the two proteins ricin and mistletoe lectin were observed which did not make it possible to clearly differentiate the permeability of the membranes depending on the type II RIPs added (Pohl et al., 1998a). Furthermore, Pohl et al. (1998b) observed that both ricin and mistletoe lectin were able to induce vesicle-vesicle fusions. The model of fusion induction, however, is not crucially relevant for the uptake of mistletoe lectin or ricin in vivo as membrane fusions are not made responsible for the uptake of these proteins by a target cell. In 1990, Tonevitsky et al., already showed that the ganglioside GM1 could not be regarded as the receptor. Utsumi et al. (1987) reported on a binding of ricin to GM1-containing liposomes, which could not be observed when the competitor lactose was added.

[0014] Samal et al. (1995) observed that mistletoe lectin, like ricin, aggregates with blood platelets. According to this analysis, mistletoe lectin does however not aggregate liposomes isolated from blood platelets, said liposomes being aggregated by ricin. The authors did not discuss a potential receptor for these lectins.

[0015] In 1994, it was observed that the changed surface structure of degenerate cells can be utilised (Gottstein et al., 1994; Usui & Hakomori, 1994). Immunotoxins, for example, were suggested for a therapy, said immunotoxins consisting of monoclonal antibodies against specific glycolipid or glycoprotein structures, which are preferably present on the degenerate cells only, and the toxic component of ricin (ricin A-chain) (Goffstein et al., 1994; Usui & Hakomori, 1994). This model emphasises the relevance of a cell-specific vehicle which makes a transport of a toxin into a target cell possible.

[0016] In the case of ricin, the carbohydrate-binding B-chain does not fulfil the requirements of such a vehicle as a receptor which has not been clearly identified but which occurs ubiquitously has been described for ricin. This observation explains the side-effects described for treating patients with ricin.

[0017] As mistletoe lectin, in contrast to ricin, seems to bind to a receptor which does not occur ubiquitously, the B-chain was suggested as possible vehicle for a transport of fused toxins. Corresponding fusion proteins of the B-chain of the rViscumin having cytotoxic compounds were described in EP application EP 1012256 A1. For an efficient and targeted application of corresponding therapeutic agents, it would thus be desirable to identify the receptor which the B-chain of the mistletoe lectin or the recombinantly produced rViscumin binds to.

[0018] The use of mistletoe extracts (extracts of *Viscum album*) as a remedy has already been known for centuries. Ingredients in this case called lectins are identified as active components of these extracts. These lectins are proteins which can recognise very specific carbohydrate structures even in lipid- or protein-bound form and can bind thereto. Mistletoe lectin, which was characterised as class II ribosome-inactivating protein, is pharmacologically effective

only thanks to the interaction of its two sub-units. In this case, the B-chain of the mistletoe lectin which has sequence motifs with specific carbohydrate-binding properties, is responsible for the transport of the protein into the target cell. In the target cell, the A-sub-unit then blocks the ribosomal metabolism in the cell by its enzymatic rRNA-N-glycosidase activity and, in this way, triggers a programmed cell death (apoptosis) in said cell.

[0019] The mode of action of the mistletoe plant and the extracts obtained therefrom for treating diseases was described in European patent EP 0 602 686 B1. Since the beginning of this century, mistletoe preparations are used in cancer therapy with mixed success (Bocci, 1993; Gabius et al., 1994; Gabius & Gabius, 1994; Ganguly & Das, 1994). Hajto et al. (1989, 1990) were able to show that the therapeutic effects are mediated in particular by so-called mistletoe lectins (viscumins, *Viscum album* agglutinins, VAA). Apart from the cytotoxic effect, it is today in particular an (unspecific) immunostimulation that is discussed, the positive effects of which are utilised for an accompanying therapy and for the follow-up treatment of tumour patients. An improvement of the quality of life of such patients is possibly brought about by the release of endogenous endorphins (Heiny and Beuth, 1994).

[0020] Numerous in vitro analyses (Hajto et al., 1990; Männel et al., 1991; Beuth et al., 1993) and in vivo analyses (Hajto, 1986; Hajto et al., 1989, Beuth et al., 1991; Beuth et al., 1992) and clinical studies (Beuth et al., 1992) prove the increased release of inflammatory cytokines (TNF- α , IL-1, IL-6) mediated by the mistletoe lectin and an activation of the cellular components of the immune system (TH-cells, NK-cells).

[0021] Today, a 60 kDa-mistletoe lectin protein is regarded as active principle of the mistletoe extracts wherein the protein can be obtained from extracts by means of biochemistry (Franz et al., 1977; Gabius et al., 1992). The ML-protein consists of two covalently S-S-linked sub-units wherein the A-chain of the protein is responsible for an enzymatic inactivation of ribosomes (Endo et al., 1988) and the B-chain of said protein is responsible for the carbohydrate binding. The biological activity is correlated with obtaining the lectin activity of the B-chain (Hajto et al., 1990).

[0022] The technical problem underlying the present invention is that it has so far not been possible to make a targeted prediction as to whether a therapy including the administration of rViscumins or similar compounds is suitable for treating a disease or ailment or an individual.

[0023] This technical problem has been solved by the embodiments characterised in the claims.

[0024] Thus, the present invention relates to an in vitro method for the determination of the responsiveness of an individual to mistletoe lectin or to (a) mistletoe lectin single chain(s), comprising the step of the specific quantitative and/or qualitative detection of a membrane-bound receptor, wherein the receptor is characterised by a terminal N-acetylneuraminic acid (Neu5Ac) which is linked to a galactose (Gal) by a glycosidic α 2-6 bond.

[0025] Thus, according to the invention, an in vitro method for the determination of the responsiveness of an individual to mistletoe lectin or for (a) mistletoe single chain is preferably described wherein the responsiveness is medi-

ated by the specific binding of the carbohydrate-binding sub-unit of the mistletoe lectin to a membrane-bound receptor and the receptor is characterised by a terminal N-acetylneuraminic acid (Neu5Ac) which is linked to a galactose (Gal) by a glycosidic α 2-6 bond. The determination comprises the specific quantitative and/or qualitative detection of said specific glycosylation.

[0026] The term "mistletoe lectin" comprises both the natural mistletoe lectins described herein and known to the skilled person and the recombinant mistletoe lectins wherein the aforementioned mistletoe lectin single chain preferably comprises the mistletoe lectin B-chain or (a) fragment(s) thereof.

[0027] In connection with the invention, the term "responsiveness" defines the triggering of a reaction of a single cell, a cell population, a cluster of cells, a tissue, an organ or an organism, said reaction favouring the healing of a disease or an ailment. Furthermore, the term "responsiveness" also comprises the sensitivity of an individual cell, a cell population, a cluster of cells, a tissue, an organ or an organism in preventive application of the rViscumins or similar compounds. Accordingly, a responsiveness of corresponding target cells is connected with a positive therapeutic effect or a preventive effect. Preferably, the "responsiveness" comprises the sensitivity of a human cell, cell population, cluster of cells, tissue, organ or a human organism.

[0028] The term "specific binding" can, for instance, be characterised by a "key-keyhole principle". The ligand (mistletoe lectin) and the target molecule (membrane-bound receptor) have structures or motifs which specifically fit each other. An example hereof are an antigenic determinant (epitope) which interacts with the antigen binding site of an antibody. Accordingly, a specific binding is in contrast to a more universal, unspecific binding. When the structure of an interaction partner that specifically bind to each other is known, conclusions as to possible preferred structures or special structural elements of a suitable partner interacting therewith can be drawn. The invention provides the specific interaction partner for mistletoe lectin, in particular rViscumins.

[0029] The term "carbohydrate-binding sub-unit of the mistletoe lectin" describes the sequence motifs of the B-chain of the mistletoe lectin which specifically bind to the membrane-bound receptor of the mistletoe lectin. These motifs were described by Langer et al. (2000). Like the carbohydrate-binding sub-unit of the ricin, the B-chain of the mistletoe lectin, too, is formed of 2 domains. These domains are again divided into 3 sub-domains each. The domains are designated as 1 and 2, the sub-domains as α , β and γ . For ricin, it is described that every sub-domain is derived from a presumably bacterial carbohydrate binding structure (Rutenber et al. 1987). Due to the high structural identity of the B-chain of the ricin and the mistletoe lectin (62% identity and 70% homology according to Eck et al., 1999), this observation also seems to be applicable to the mistletoe lectin. According to Langer et al. (2000), the different specificity of the carbohydrate-binding sub-unit of the ricin and the mistletoe lectin is due to the differences in the sub-domains. These are, in particular, in the 1 α sub-domain the residues D23 and W38, in 1 β the residues Y68, Y70, Y75 and F79 and in sub-domain 2 γ the residues D235, Y249; for numbering, cf. Eck et al. (1999a).

[0030] In connection with the invention, the term “membrane-bound receptor” defines a membrane-bound structure which is characterised by a terminal N-acetyl neuraminic acid (Neu5Ac) which is again linked to a galactose (Gal) via a glycosidic α 2-6 bond. Examples of corresponding membrane-bound structures are proteins or peptides anchored to the membrane of a cell. This definition comprises both transmembrane and membrane-associated proteins and peptides. Lipids which are either themselves part of the membrane or which are associated thereto are also examples of such structures. The membrane-bound structure disclosed can be part of both a glycoprotein (glycosylated protein) and a glycolipid.

[0031] In this connection, the term “membrane” explicitly comprises all membranous, cellular lipid double layers. Accordingly, both membranes of the endoplasmic reticulum (ER), Golgi apparatus, the nuclear envelope, of vesicles and vacuoles and the outer cell membrane.

[0032] In the state of the art, proteins are described which are glycosylated with a terminal N-acetyl neuraminic acid (Neu5Ac) which are linked to a galactose (Gal) via a glycosidic α 2-6 bond. These glycosylated proteins, however, are soluble proteins/serum proteins (cf., e.g. Hanasaki et al., 1995) which are neither relevant for the pharmacological effects nor the mode of action of mistletoe lectin. In connection with this invention, a glycosylated membrane-bound structure is disclosed for the first time.

[0033] The identification of terminal N-acetyl neuraminic acid (Neu5Ac), which is linked to a galactose (Gal) via a glycosidic α 2-6 bond, as a specific target structure of the carbohydrate-binding sub-unit of the mistletoe lectin allows a skilled person, either alone or in combination with the teaching of Langer et al. (2000), for instance, to change the carbohydrate-binding sub-unit of the ricin by site-specific mutagenesis. A peptide/protein mutated accordingly could then no longer bind to a ricin-specific target structures but to mistletoe lectin-specific target structures instead.

[0034] Examples of a quantitative and/or qualitative detection of said glycosylation are known to the person of skill in the art and are described, amongst others, in the enclosed example 7 and in FIG. 9 which illustrates this example. Such methods comprise, for instance, modified Western blot analyses as shown in the examples. Moreover, such methods comprise techniques such as, e.g. the radioimmunoassay (RIA), sandwich (immunometric assay) and Western blot assay, IRMA (immune radioimmunometric assay), EIA (enzyme immunoassay), ELISA (enzyme-linked immunosorbant assay), FIA (fluorescent immunoassay), CLIA (chemiluminescent immunoassay), agglutination assay and flow cytometric methods.

[0035] The method of the invention allows, inter alia, a prognosis on the effectiveness of a mistletoe lectin therapy, which includes that a forecast can be made whether a therapy with mistletoe lectin, preferably with rViscumin, can principally be expected to be successful in the treatment of a disease in certain cells, a certain cell population or a certain tissue, organ or organism. If, for example, the aforementioned glycosylation on a tumour cell is identified using the method of the invention, rViscumin can bind to the cell, be absorbed by it and exert a cytotoxic effect in it. Moreover, the method of the invention allows a prognosis of the effectiveness of a mistletoe lectin therapy in an individual.

As a consequence, in a group of patients, individual responders (patients responding to the therapy) can be differentiated from non-responders. Such a method of prognosis therefore fulfils a similar task as examinations which the skilled person knows and which are carried out prior to a therapy of breast cancer patients with Herceptin. Before a therapy with the antibody preparation Herceptin is allowed to be administered, it must be tested whether a patient has the EGF-receptor Her-2. In this connection, the FDA (US Food and Drug Administration) has authorised in particular two test methods (immunohistochemistry staining (IHC) and fluorescence in situ hybridisation (FISH)). Cf. in this connection amongst others Thomson et al. (2001). Analogously, such methods and other techniques described herein can be used, according to the teaching of the invention, for recognising and/or determining individual responders to a mistletoe lectin therapy, in particular a therapy with rViscumin.

[0036] For the method of the invention, samples of fluids, in particular body fluids, cells, cell populations or tissues can be used. These samples can be derived from blood samples or other samples of body fluids but can also be tissue samples, individual cells or disseminated tumour cells. Examples of such samples and techniques of how to take them are described in more detail in this application in connection with further embodiments.

[0037] The method of the invention also makes it possible to recognise responders in a group of patients early. In this way, it is possible to assess the success of the therapy in a test that can be readily carried out without great delay prior to the beginning of a therapy. This characteristic of the method of the invention is relevant particularly with regard to aspects of disease economy. A possibly cost-intensive therapy which however is ineffective for the individual patient is avoided. In this way, also side effects which are tolerated in the case of an effective therapy if these individual patients for which a successful therapy with rViscumin seems doubtful can be avoided.

[0038] As described in the examples, in tests underlying the invention, it could clearly be shown that mistletoe lectin and ricin bind to glycosphingolipids (GSL). It was for the first time possible to document in this case a quantitative and qualitative difference between the binding specificity of mistletoe lectin (in particular rViscumin) and ricin. The carbohydrate structures recognised by the two proteins are schematically shown in FIG. 1.

[0039] As is shown in the illustrated examples, it was shown in specific tests that the primary specificity of mistletoe lectin (in particular rViscumin) is not a terminal galactose. FIGS. 2 to 5 and 7 show a modified Western blot/immunologic detection of bound ricin or rViscumin on a DC plate (TLC assay) on which the neutral and acidic GSL have been separated beforehand due to their different running properties in different running agents. While ricin shows a clear specificity for neutral GSL with terminal galactose (Lc2, nLc4, nLc6, Gg4) (table 1; FIG. 4B, lane b), rViscumin surprisingly only shows a very weak binding to Gal β 1-4Glc β 1-1Cer (Cer means in this case ceramide) (table 1; FIG. 2A, lane b, negative stain after overnight incubation).

[0040] Surprisingly, it was shown that this does however not apply to a group of gangliosides at which an N-acetyl

neuramic acid (Neu5Ac) is located terminally which is recognised very well by rViscummin and not at all by ricin (cf. table 1; FIG. 2B, lane b; FIG. 5B, lane b, negative stains). Moreover it was surprisingly found that it is crucial for the recognition of the receptor structure by rViscummin whether the N-acetyl neuramic acid is linked to the terminal galactose of the neutral sugar structure α 2-6 (is recognised) or α 2-3 (is not recognised) (cf. enclosed examples and figures).

[0041] This inventive, specific recognition of N-acetyl neuramic acid in Neu5Ac- α 2-6-Gal configuration reminds of the specificity in the epitope recognition of monoclonal antibodies and has not been described so far for mistletoe lectin. The absolutely different specificity of ricin and rViscummin, too, was surprising and not predictable for the skilled person. The different recognition motifs of the two type II-ribosome-inactivating proteins tested are summarised in table 1. It could be concluded from the state of the art that both ricin and mistletoe lectin (in particular rViscummin), if at all, rather bind to the neutral GSL which have a galactose as terminal sugar residue.

[0042] In a preferred embodiment of the method, the receptor comprises gangliosides which, after the α 2-6-sialylated galactose, at least one N-acetyl glucosamine (GlcNAc), i.e. gangliosides which, after the α 2-6-bound N-acetyl neuramic acid to galactose, comprise at least one N-acetyl glucosamine (GlcNAc). As explained below, the following N-acetyl glucosamine (GlcNAc) can however be located both directly and indirectly after the Neu5Ac α 2-6-Gal structure. Thus, in connection with this invention, it is possible that the method of the invention is based on the (structure) recognition of a ganglioside receptor comprising the structure Neu5Ac α 2-6-Gal-Y-[GlcNAc]_x-Cer. Y can, for instance comprise a further galactose (Gal).

[0043] In connection with the invention, the term "ganglioside" defines acidic sialic acid containing ceramide oligosaccharides. The carbohydrate moieties are linked via a glycosidic bond to the C1-OH group of N-acyl sphingosine (=ceramide). Gangliosides can be both long-chain (e.g. IV³nLc4, VI³nLc6, etc.) and branched (GM1 or GM2), (Voet and Voet (1992), in particular the figure on page 271 of this reference).

[0044] In a moreover preferred embodiment of the method, the receptor comprises gangliosides with the structure Neu5Ac α 2-6-Gal-[Gal/GlcNAc]_x-Cer wherein Cer is a ceramide. In the method described herein, Neu5Ac α 2-6-[Gal β 1-4GlcNAc β 1-3]_x Gal β 1-4Glc β 1-1Cer is particularly preferred.

[0045] Common methods for the structural characterisation of gangliosides, at present only allow an exact analysis of gangliosides where x is \leq 6. The carbohydrate-binding sub-unit of the mistletoe lectin recognises the terminal sugar structures. Accordingly, the value of the variable x is principally not important for the specific binding. Preferably, the variable has a value of 10 at maximum. Moreover, the variable preferably has a value of \leq 6, more preferably a value of 5, 4, 3, 2 or 1. Furthermore, it is preferred that, in the case of branched gangliosides, the value of x is different in individual or all chains.

[0046] It is also preferred that, in an embodiment of the method, the receptor comprises gangliosides having the structure Neu5Ac α 2-6-[Gal1-4GlcNAc β 1-3]_x Gal β 1-

4Glc β 1-1Cer, the structure Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer being particularly preferred, or a ganglioside Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer.

[0047] This preferred embodiment, too, comprises linear and branched gangliosides.

[0048] According to a further preferred embodiment, the receptor is cell membrane-bound.

[0049] This embodiment of the method of the invention describes the analysis of membrane-bound receptors which form part of the outer cell membrane or are bound thereto. In particular, said embodiment comprises receptors that are glycoproteins or glycolipids.

[0050] In a further preferred embodiment, the mistletoe lectin is a recombinant mistletoe lectin/rViscummin.

[0051] As described above, in the literature a difference is made between various naturally-occurring mistletoe lectins (ML-I, -II and -III). They differ in their carbohydrate specificity (Franz, 1986). In this case, it is discussed that the specificity of galactose/lactose (ML-I) changes from a mixed form of galactose/lactose and N-acetyl galactosamine (ML-II) to a mistletoe lectin which binds more strongly to N-acetyl galactosamine (ML-III). The skilled person knows recombinant mistletoe lectin/rViscummin provided in *E. coli* (EP 0 751 221 B1; Eck et al., 1999a and 1999b). The primary structure of rViscummin does not exactly correspond to an ML-I, ML-II or ML-III that can be found in the mistletoe plant. It can be assumed that, apart from the rViscummin sequence described, there are other mistletoe lectin variants modified by other site mutations in the original gene, which may slightly differ in their primary structure. rViscummin could be regarded as a variant of the primary mistletoe lectin and could be understood as a mixture of ML-I, ML-II and ML-III sequences.

[0052] Moreover, this embodiment comprises recombinant fusion proteins on the basis of the rViscummin as described in EP patent application EP A2 1012256.

[0053] Furthermore, it is preferred that, in one embodiment of the invention, the recombinant mistletoe lectin comprises an amino acid sequence which is encoded by a polynucleotide as shown in SEQ ID No.: 1. SEQ ID No.: 3 or SEQ ID No.: 5.

[0054] A further preferred embodiment preferably also comprises a recombinant mistletoe lectin encoded by one or more polynucleotides.

[0055] In a preferred embodiment of the method of the invention, the recombinant mistletoe lectin also comprises a polypeptide with an amino acid sequence as shown in SEQ ID No.: 2, SEQ ID No.: 4 or SEQ ID No.: 6 or a functional fragment thereof.

[0056] In connection with this invention, the term "functional fragment" defines fragments of the polypeptides mentioned which have the same biological function as the polypeptides shown with an amino acid sequence (SEQ ID). The function of mistletoe lectin, in particular rVisummin, and the known sub-units of the mistletoe lectin has already been described herein. The "function" also comprises the specific binding property disclosed herein of the mistletoe lectin, in particular of the B-chain of the mistletoe lectin, for the

mistletoe lectin receptor described. Thus, fragments of the mistletoe lectin B-chain, in particular fragment which can mediate a specific interaction with the mistletoe lectin receptor described are comprised, too.

[0057] In this context, the term “same biological function” describes for instance that, e.g. fragments or derivatives of the polypeptides induce the same signals in a cell as the peptides mentioned. Examples of fragments are peptide domains with defined functions or specific prosthetic groups. The “same biological function” also comprises the cytotoxicity, immunostimulation (both of the native and the adaptive immune system), stimulation of the release of cytokines, antigenicity, induction of the expression or the activation of surface markers (e.g. CD56 on NK cells), induction of apoptosis or endorphin stimulation.

[0058] In a further preferred embodiment, recombinant mistletoe lectin is also comprised which is encoded by one or more polynucleotides which encode a polypeptide with an amino acid sequence as shown in SEQ ID No.: 2, SEQ ID No.: 4 or SEQ ID No.: 6 or a functional fragment thereof, the sequence of which is however degenerate when the genetic code is taken into consideration.

[0059] The mistletoe lectin receptor described herein for the first time also plays a role in cellular tests as described in the examples. It could be shown that cells react differently to a treatment with rViscumin. In particular tumour cells or cells derived from tumour cells show, as described in the examples, specific biochemical reactions to the treatment with rViscumin.

[0060] By the experiments described in the examples, it was also possible to clearly identify the specificity of rViscumin for the receptor disclosed herein. Moreover, it was clearly shown that rViscumin has a quantitatively different carbohydrate specificity compared to ricin.

[0061] In the experiments underlying the invention and described in the examples, it was shown that tumour cells or tumour cell lines express the aforementioned specific receptor for mistletoe lectin, in particular rViscumin.

[0062] An alternative embodiment of the method of the invention is an in vitro method for the determination of a responsiveness to mistletoe lectin(s) which comprises the quantitative and/or qualitative determination of the sialyltransferase(s).

[0063] Sialyltransferases are responsible for the terminal glycosylation of glycosylated structures in eukaryotic cells. The activity of these enzymes was found in the Golgi apparatus (in the trans-Golgi compartment). The enzymes catalyse the transfer of sialic acid residues. The enzyme activity of such sialyltransferases catalyses, for instance in degenerate cells (cancer cells), a specific glycosylation of peptides and/or lipids. A presence of such sialyltransferases in cell or tissue samples can be considered an indication of a degeneration of cells. This holds even more true for the detection of an enzymatic activity of these sialyltransferases in a sample.

[0064] Methods of molecular biology and protein biochemistry for the quantitative and/or qualitative determination of sialyl transferase(s) are known to the skilled person from the literature (cf. amongst others, Mülhardt (2000) and Rehm (2000)). Methods of molecular biology are for

instance RT-PCR techniques, RNase projection assays, Northern blot or Southern blot analyses. Methods of protein biochemistry are for instance methods for detecting a transferase activity but also methods such as Western blot analysis or other techniques that can be summarised in the term “proteom analysis”.

[0065] Furthermore, the quantitative determination of the sialyl transferase of individual cells can for example comprise disseminated tumour cells. Individual cell analyses are known to the skilled person and described in EP A1 11009938 and Klein (1999).

[0066] An example of a corresponding sialyl transferase is the β -galactoside α -2,6-sialyltransferase E.C. 2.4.99.1 (α 2-6STN). α 2-6STN is a 47 kDa-transmembrane protein. Hepatocytes also secrete a 41 kDa-form of said enzyme. Soluble α 2-6STN is a serum glycoprotein which is assigned to the group of acute phase reactants and plays a role in pathologic processes (enhanced activity in many carcinomas, e.g. colon carcinoma and cervical carcinoma). The amino acid sequences of the known forms of the enzyme and the nucleotide sequences encoding them are known to the person of skill in the art (cf. accession numbers L29554 (*Rattus norvegicus*), X75558 (*Gallus gallus*), NM_003032 (*Homo sapiens*) and NM_009175 (*Mus musculus*)).

[0067] The methods according to the invention, which are presented herein, can be carried out using a specific detection reagent. Such specific detection reagents are capable of interacting with or binding to the mistletoe lectin receptor described herein. Said interaction or binding can be direct or indirect, should however be specific for the receptor described herein. Particularly preferred reagents herein are specific antibodies, antibody fragments or antibody derivatives, aptamers, carbohydrate-binding molecules (e.g. peptides and/or proteins) wherein the reagent has to be capable of recognising and/or binding the mistletoe lectin receptor described herein or its specific components (e.g. the terminal N-acetyl neuramic acid defined herein and linked to a galactose via a glycosidic α 2-6 bond). Preferably, these detection reagents are labelled wherein the label can comprise radioactive substances, fluorescence dyes, biotin-(strept)avidin, luciferases, CAT, beta-galactosidase, alkaline phosphatase(s), peroxidase(s), further enzymatic labels, digitonin, dyes in general, etc. The method of the invention can however also be carried out by means of an indirect detection of the receptor described herein. Detections of the receptor are illustrated in the examples and can, as mentioned above, also comprise methods such as RIA, ELISA, CLIA, FIA, ELLA, TLC tests, histological methods, direct and indirect (immuno) fluorescence methods or flow-through methods (e.g. FACS analyses, flow cytometry, BIA-core).

[0068] In an alternative embodiment, the invention relates to a diagnostic composition comprising a substance which specifically recognises or binds to an aforementioned receptor, preferably selected from the group of antibodies, antibody derivatives, antibody fragments, aptamers and carbohydrate-binding peptides or proteins. According to the invention, however, also lectins, e.g. labelled lectins, could be used for diagnostic purposes. In this case, in particular, labelled lectins are used which are capable of specifically binding to the receptor described herein.

[0069] Methods for producing antibodies (polyclonal or monoclonal) which recognise or bind a specific target struc-

ture are known to the skilled person. The production of monoclonal antibodies against the gangliosides GD1 and GD2, which play a role in neurological diseases, was described by Magnani et al. (1982), Tur et al. (2001) and Pan et al. (2001). The detection of the specificity of such antibodies can, amongst others, can [. . .]₁ by overlay assay which is based on an immune staining after thin-layer chromatography (Müthing and Mühlradt (1988), Müthing and Kemminer (1996), Müthing (1998)). Said method can optionally also be combined with ELISA.

¹ translator's note: verb missing in the original

[0070] The antibodies to be used in the method described herein are preferred to be monoclonal antibodies (or fragments or derivatives thereof) which specifically bind to the membrane-bound receptor structure disclosed herein. In the examples, it is shown how to obtain such antibodies. For example the antibodies described in the examples designated 59.33.3, 59.33.5 and 59.33.6 are directed against the α -2-6 sialylated neolacto-type-ganglioside described herein, i.e. the receptor for (recombinant) mistletoe lectin described herein.

[0071] The antibodies can, amongst others, be antibodies of the type IgG, IgA, IgD, IgM. A particularly preferred antibody can be obtained from an hybridoma/hybridoma cell line which was deposited with the Deutsche Sammlung von Mikroorganismen und Zellkultureno GmbH (DSMZ) in Brunswick on 20 Dec. 2002 under the designation 59.33.3 under the accession number r [. . . number] according to the Budapest Treaty.

[0072] It is also provided for that the diagnostic and pharmaceutical compositions described herein (see below) comprise antibodies which are obtained from the antibodies described herein by modifications of the same. In this way, by means of techniques known to the skilled person, the CDR (complementarity-determining regions; the three complementarity-determining regions CDR1, CDR2 and CDR3 are loops at the end of a V-domain of antibodies or T-cell receptors. They get into direct contact with an antigen or a peptide: MHC complex) of the antibodies described herein can be isolated and said CDR can be inserted in antibodies having a different structure. Thus, it is possible to isolate the CDR from the 59.33.3 antibody (an IgM) and to integrate this CDR in other immunoglobulins, e.g. an IgG, preferably in an IgG1 structure. These methods (e.g. CDR-grafting) are well known to the skilled person. Therefore, the antibodies described herein also comprise chimeric antibodies, humanised antibodies or single-chain antibodies or single-chain antibody fragments such as scFv constructs. However, such antibody modifications are not limited to modifications of the antibodies 59.33.3, 59.33.5 or 59.33.6 described in detail herein. Antibodies to be used can be all antibodies/antibody molecules, antibody fragments or antibody derivatives which specifically bind to the receptor described herein for (recombinant) mistletoe lectin ((r)Viscumin).

[0073] Antibody fragments such as Fv, F(ab')₂ and Fab can be produced by cleaving the intact protein (antibody), e.g. by protease or by chemical cleavage. A truncated gene may, however, also be designed. A chimeric gene, for instance, which encodes a part of the F(ab')₂ fragment comprises DNA sequences which encode the CH1 domain and hinge region of the H-chain followed by a translational stop codon, which results in a truncated molecule.

[0074] The term "antibody derivatives" describes modified forms of the aforementioned antibodies. These modifications comprise chemical, in particular biochemical modifications, preferably modifications of protein biochemistry, of the antibodies or their fragments. In this case, the common feature which characterises the antibodies, antibody fragments and their derivatives is the recognition of and/or binding to a specific target structure. According to the invention, said target structure is the membrane-bound receptor for mistletoe lectin described herein. Aptamers and their specific properties have been described as a summary, amongst others, by Hermann and Patel (2000). The skilled person knows methods for isolating aptamers from the state of the art, too.

[0075] Herein, the term "carbohydrate-binding peptides" is defined as in the embodiment of the invention which relates to a use for producing a pharmaceutical composition.

[0076] In a further preferred embodiment of the diagnostic composition, the substance is detectably labelled.

[0077] Examples of detectable labellings of substances are known to the person of skill in the art. They include, amongst others, radioactive, enzymatic or fluorescent labels. Biotinylation and a detection using avidin or streptavidin is also an example of a corresponding preferred embodiment.

[0078] An embodiment of the invention comprises the use of the diagnostic composition according to the invention for analysing whether individual cells, a cell population or a union of cells, cells in a tissue, organ or organism have a functional receptor for mistletoe lectin.

[0079] In a further alternative embodiment, the invention also relates to the use of an above-defined substance for the preparation of a diagnostic agent for detecting a functional mistletoe lectin receptor.

[0080] The use according to the invention of the diagnostic agent for the detection of the receptor which is described herein and which is examined in the examples is moreover preferred on cells.

[0081] As shown in the examples, tumour cells which express terminal N-acetyl neuraminic acids (Neu5Ac), which are linked to galactose (Gal) via a glycosidic α -2-6 bond, are particularly suitable as responders to mistletoe lectin, particularly to recombinantly produced mistletoe lectin (rViscum).

[0082] In a preferred embodiment the examined cells are tumour cells.

[0083] More preferred these tumour cells are animal cells, preferably tumour cells derived from mammals, furthermore preferred tumour cells derived from humans. Furthermore preferred these cells are leukaemic cells, microcellular or non-microcellular lung carcinomas, colon carcinomas, CNS carcinomas, melanoma, ovarian carcinomas, kidney carcinomas, prostate carcinomas, mamma carcinomas, bladder carcinomas, gastric carcinomas, pancreatic carcinomas or carcinomas of the testicle.

[0084] In a further preferred embodiment of the invention the cells are derived from biopsy material.

[0085] Methods for the recovery of biopsy material are known to the skilled person. This description comprises different recovery types, where cells are taken from a

proband or patient. Cells which are living cells are preferred. These are for example biopsies carried out with fine needles (fine needle aspirations), punch biopsies, the removal of solid tissue parts (e.g. by surgical methods) or samples of entire organs (e.g. adrenal glands). The different removal techniques are e.g. described in: Kremer et al (1999), Pichlmayr and Löhlein (1991), Niethard and Pfeil (1997), Malte (1998) and Bonk.

[0086] In an also preferred embodiment the cells are isolated from blood samples.

[0087] Apart from the isolation of cells from blood samples, also preferred embodiments of the invention refer to the isolation of cells from pleural effusions, ascites samples, rinsing fluids, urine samples, sperm samples or samples from spinal and cerebral fluids.

[0088] In order to determine the responsiveness of e.g. cancer patients to rViscumin, before a planned therapy or during the course of the therapy, tissue is taken from the patients. It is also possible to take recourse to collected samples/preserved samples of (different) patients (which are stored in many hospitals in paraffin block banks which are arranged according to the indications) in order to be in a position to make a statement with respect to a specific indication which has a sound statistic basis.

[0089] These tissues are e.g. lyophilised, preserved in formaldehyde solution or obtained by cryoconservation (purposeful freezing, e.g. after persution with a cytoprotective solution, e.g. sugar solution) and are subsequently further processed according to methods known to the skilled person, e.g. preserved in paraffin blocks. Starting from the samples, slices are prepared which are suitable for an examination with a microscope. Subsequently, these slices are then examined with methods which are well-known to the skilled person (e.g. the LSAP method, a method similar to ELISA or by using the ENVISION method of DAKO in Hamburg) for the presence of the specific antigens, i.e. of the receptor for (recombinant) mistletoe lectin defined herein. To this avail, e.g. samples from patients can be used by using the mAb 59.33.3 or the two other mAbs 59.33.5 or 59.33.6 which specifically recognise the epitope "CD75s" of the receptor described herein. Bound antibodies which can e.g. be used in a concentration of 500 ng/ml up to 10 µg/ml, are then detected with secondary antibodies or other detection methoAan anti-mouse IgM antibody to which polydextrane is linked which is provided with alkaline phosphatase can, e.g. be used. Thus, a comparison between healthy and degenerate tissue is possible as well as a differentiation whether in the case of a patient or in the case of a specific tumour indication a treatment with rViscumin is promising. Moreover, it can be detected whether a treatment with (recombinant) mistletoe lectin ((r)Viscumin) is successful, i.e. the course of the therapy can be monitored.

[0090] Moreover, the invention described herein makes other diagnostic methods such as, inter alia, the examination of fine needle biopsy material and xenograft cell lines or other cell lines possible.

[0091] The cells can, e.g., be fixed in 96-well plates and can be examined for the presence of membrane-bound/membrane-stable CD75s motifs (rViscumin-binding motifs, i.e. of the membrane-bound receptor structure described herein) in a method similar to ELISA, as described in

example 8. Another method which can be used for the examination is the FACS analysis. Here, cells can be taken from a pool of cells (interesting particularly in the case of fine needle biopsies) which carry a certain surface antigen. Thus, the cells are at first, e.g., used by using specific antibodies or antibody derivatives such as the mAbs 59.33.3, 59.33.5 or 59.33.6 described herein. Bound antibodies which can preferably, but not excludingly, be used in a concentration of 500 ng/ml to 10 µg/ml, are then placed in an automatic Cell Sorter e.g. by Becton Dickinson together with a secondary antibody, e.g. an anti-mouse IgM antibody which is (fluorescence-) labelled with FITC or otherwise. In this method, in order to filter a specific cell population, a second epitope present on the target cells can be marked with an antibody which is different from the (fluorescence-) labelled anti-mouse IgM antibody and thus only the cells which are in this case labelled twice can be specifically selected. This provides other possibilities to predict the patient with respect to a treatment with rViscumin. Additionally, the information how many percent of a corresponding cell population (cells stained twice vs. cells who are only stained with the second antibody) carry the desired CD75s epitope can be obtained. From such an analysis it can be assessed or taken whether a minimal residual disease exists. Additionally, combination therapies with other anti-tumour agents together with rViscumin can be tested in vitro and can be prepared for the application to a patient.

[0092] The diagnostic method as described herein does, however, also comprise the analysis of lysed membrane structures. It is, inter alia, possible to e.g. treat fresh, unfixed biopsy material with detergents (e.g. Triton X-100®, Triton X-112®, Tween 80®, Tween 20®, Octylglycosid, etc.) and to analyse the lysate e.g. by Western blotting or in the form of an ELISA/RIA test. This means that the detergent lysates can be tested for the original presence of the herein described membrane-bound receptor for (recombinant) mistletoe lectin ((r)Viscumin). The skilled person has sufficient methods for the corresponding analysis.

[0093] In a further alternative embodiment, the invention refers to the use of a substance which specifically binds to or recognises one of the receptors defined above for the preparation of a pharmaceutical composition for the treatment of proliferative diseases (e.g. cancer), viral diseases (e.g. herpes, HIV), autoimmune diseases or neuronal diseases wherein the receptor-binding or receptor-recognising substance is selected from the group of antibodies, antibody derivatives, antibody fragments, aptamers, low-molecular substances and carbohydrate-binding peptides. Preferably, the receptor-binding or receptor recognising substance is capable to inhibit or weaken a binding of the B-chain of mistletoe lectin and/or rViscumin to this receptor.

[0094] In the context of the invention, the term "inhibit" describes a complete as well as a partial inhibition. Thus, the term comprises a total blocking of the binding of mistletoe lectin and/or rViscumin to the carbohydrate structure which has been identified as specific receptor. Moreover, the ability to weaken the binding of mistletoe lectin and/or rViscumin to the receptor is also comprised.

[0095] The substance described above for the preparation of a pharmaceutical composition is not mistletoe lectin and particularly not the B-chain of mistletoe lectin.

[0096] This embodiment of the invention moreover preferably refers to the formulation of pharmaceutical compo-

sitions, possibly in combination with a "pharmacologically acceptable carrier" and/or a diluent. Examples of particularly suitable pharmacologically acceptable carriers are known to the skilled person and comprise buffered saline, water, emulsions such as e.g. oil-in-water emulsions, different kinds of detergents, sterile solutions, etc. Pharmaceutical compositions comprising such carriers can be formulated with the help of known conventional methods. These pharmaceutical compositions can be administered to an individual in a suitable dose. The administration can be carried out orally or parenterally, e.g. intravenously, intraperitoneally, subcutaneously, intramuscularly, locally, intranasally, intrabronchially or intradermally or via a catheter at a site in an artery. The type of dosage is determined by the physician in charge depending on the clinical factors. The skilled person knows that the type of dosage depends on different factors such as e.g. height or weight, body surface, age, sex or the general condition of the patient, but also on the substance which is to be specially administered, the duration and type of administration and on other medicaments which are possibly administered at the same time. A typical dose can e.g. be in the range of between 0.001 and 1,000 μg , with doses below or above of this exemplary range are thinkable, especially when taking the above-identified factors into consideration. Generally, in the case of a regular administration of the composition of the invention, the dose should be in the range between 10 ng and 10 mg units per day or per application interval. If the composition is administered intravenously, the dose should be in a range of between 1 ng and 0.1 mg units per kg body weight per minute.

[0097] The composition can be administered locally or systemically. Preparations for a parenteral administration comprise sterile aqueous or non-aqueous solutions, suspensions and emulsions. Examples of non-aqueous diluents are propyleneglycol, polyethyleneglycol, plant oils such as e.g. olive oil, and organic ester compositions such as e.g. ethyl-oleate, which are suitable for injections. Aqueous carriers comprise water, alcoholic-aqueous solutions, emulsions, suspensions, salines and buffered media. Parenteral carriers comprise sodium chloride solutions, Ringers dextrose, dextrose and sodium chloride, Ringer's lactate and bound oils. Intravenous carriers comprise e.g. supplements for fluids, nutrients and electrolytes (such as e.g. those which are based on Ringer's dextrose). The composition of the invention can moreover comprise preservatives and other additives such as e.g. antimicrobial compounds, antioxidants, chelating agents and inert gases. Moreover, dependent on the intended use, compounds such as e.g. interleukins, growth factors, differentiating factors, interferons, chemotaktic proteins or an unspecific immunomodulatory agent can be contained. It is particularly preferred that cytostatics, antibiotics and combinations thereof can be contained. The pharmaceutical compositions or medicinal products produced can be used either for the prophylaxis or the treatment of one of the ailments or one of the diseases mentioned above.

[0098] The production and methods for the isolation of antibodies, antibody fragments, antibody derivatives and aptamers are known to the skilled person from the prior art as described above and have already been described in more detail above. According to the invention, substances which correspond to a B-chain of the mistletoe lectin or rViscumin or derivatives thereof are not comprised in the group of lectins and carbohydrate-binding peptides. However, this embodiment comprises, inter alia, other lectins which have

been adapted with respect to their binding properties to the binding properties of the B-chain for the receptor described herein by biochemical modifications and/or modifications of molecular biology (e.g. directed mutagenesis).

[0099] Moreover, in another preferred embodiment, the receptor-binding and/or receptor-recognising substance is linked to a compound with a radioactive, cytotoxic or cytostatic effect.

[0100] In this embodiment, the type of link of the substance to a compound with a radioactive, cytotoxic or cytostatic effect depends on the substance and the compound. Preferably, both are, e.g., peptides or proteins. In this case, the linking is preferably carried out by one or more peptidic bonds and/or disulfide bonds. Monoclonal antibodies (mAb) or cytokines which are labelled with radioactive substances are examples of radioimmune substances. A preferred embodiment moreover refers to linking corresponding substances with lectines, toxins or toxoids, preferably from bacteria (e.g. tetanus toxoid, tetanus toxin, diphtheria toxin, cholera toxin, *Pseudomonas* exotoxin, *Pseudomonas* toxoid, pertussis toxin, pertussis toxoid, clostridium exotoxin or clostridium toxoid or from plants (type I RIPs such as saporine or gelonine or type II RIPs such as ricin or the A-chain of type II RIPs or an A-chain of the type I RIPs which is homologous to the type II RIPs). Moreover, other low-molecular molecules with a radioactive, cytotoxic or cytostatic effect (small molecules) and other macromolecules than the ones described above are also comprised.

[0101] The term "macromolecules" refers to molecules with a high molecular complexity or a high molecular weight. These are, preferably, biomolecules, such as e.g. biopolymers, particularly proteins, oligo- or polypeptides but also DNA, RNA, oligo or polynucleotides, prosthetic groups, lipids, oligo and polysaccharides and their modifications and also synthetic molecules. The proteins preferably also comprise fusion proteins. The term peptides or proteins comprises natural and synthetic peptides or proteins. Examples of natural proteins comprise inter alia antibodies, antibody fragments, receptors which bind to their specific ligands, peptidic ligands which interact with their specific receptors or peptide domains which interact with specific substrates including proteins and coenzymes and other peptides or enzymes, etc. Moreover, recombinantly produced forms of the proteins or peptides mentioned above are also comprised here. Correspondingly, natural peptides comprise inter alia fragments of the proteins described above which interact with specific affinity ligands. Synthetic proteins or peptides comprise pseudogenes which were brought to expression or fragments thereof and proteins or peptides with a random amino acid sequence.

[0102] The term "low-molecular molecules" refers to molecules which have a lower molecular complexity than the macromolecules defined above. In the literature the term "small molecules" or "low molecular weight molecules" is not used consistently. In WO 89/03041 and WO 89/03042 molecules with molecular weights up to 7,000 g/mol are described as small molecules. Commonly, however, molecular weights of between 50 and 3,000 g/mol, more commonly, however between 75 and 2,000 g/mol and mostly in the range of between 100 and 1,000 g/mol are indicated. The skilled person knows examples from the documents WO

86/02736, WO 97/31269, U.S. Pat. No. 5,928,868, U.S. Pat. No. 5,242,902, U.S. Pat. No. 5,468,651, U.S. Pat. No. 5,547,853, U.S. Pat. No. 5,616,562, U.S. Pat. No. 5,641,690, U.S. Pat. No. 4,956,303 and U.S. Pat. No. 5,928,643. Oligomers or also small organic molecules such as oligopeptides, oligonucleotides, carbohydrates (glycosides), isoprenoids or lipid structures can be indicated as examples of such small molecules. In the literature mostly the molecular weight is the basis for the definition of such small molecules.

[0103] In another preferred embodiment of the invention, the compound described above which is linked to the receptor-binding and/or receptor-recognising substance is a peptide.

[0104] In a moreover preferred embodiment of the use according to the invention, the receptor-binding and/or receptor-recognising substance comprises a further domain which can induce an immunologic effector function.

[0105] In the context of the invention, the term "immunologic effector function" describes inter alia effector functions of the immune system which lead to the elimination of the cell which carries the receptor (target cell). The elimination is preferably an induction of programmed cell death (apoptosis), but optionally also a necrotic elimination of target cells. In vitro methods for the detection of cell death are known to the skilled person (cf. inter alia Dulat et al. (2001)).

[0106] The induction of such immunologic effector functions is preferably the induction of a signal which labels the target cell for the immune system of the organism. Preferably, the target cell is only recognised by the immune system due to this induction. It is moreover preferred that due to this induction, the cell is better recognised by the immune system. The therapy of the target cell is made possible by the recognition or better recognition of the target cell by the immune system.

[0107] In a preferred embodiment, the induced immunologic effector function is a cellular effector function.

[0108] Examples of cell-mediated immunologic effector functions are the elimination of target cells by effector-T-cells, monocytes or macrophages.

[0109] Particularly, MHC-mediated and F_c-receptor-mediated effector functions are comprised by the cellular effector function.

[0110] In an alternative preferred embodiment, the induced immunologic effector function is a humoral effector function.

[0111] Examples of humoral effector functions of the immune system are antibody-mediated reactions or reactions of the complement system. In this connection, the induction of an opsonization of target cells is a preferred embodiment of the invention.

[0112] The figures show:

[0113] **FIG. 1:** Potential structures of different receptors of rViscumin and ricin (schematically)

[0114] Schematic representation of the receptors of ricin (**FIG. 1A**) and rViscumin (**FIG. 1B**). The representation results from the interpretation of the results of the TLC

overlay assay summarised in **FIGS. 2 and 3** and **FIGS. 4 and 5**. Gal=galactose, GlcNAc=N-acetyl-glucosamine, Glc=glucose, Cer=ceramide, Sialic=sialic acid. The receptors of rViscumin are gangliosides with terminal α -2-6 linked sialic acid residues. The recognition of structures which have been recognised by ricin (**FIG. 1A**) is not carried out by rViscumin.

[0115] **FIG. 2:** TLC test for the identification of gangliosides, which are specific for rViscumin, in different cell fractions

[0116] TLC overlay binding tests of rViscumin with neutral GSL (A) and gangliosides (B) of human granulocytes. (A) Lane a: Chromatogram of 15 μ g neutral GSL (stained with Orcinol, complete sugar staining); lane b: corresponding overlay assay. (B) Lane a: Chromatogram of 15 μ g human gangliosides from human granulocytes (stained with resorcinol, sialic acid staining); lane b: corresponding overlay assay.

[0117] **FIG. 3:** TLC test for the specification of the binding specificity of rViscumin to carbohydrate on isolated gangliosides

[0118] (A) Resorcinol staining (example 1); (B) anti-IV⁶nLc4Cer antiserum TLC overlay test (example 1); (C) rViscumin TLC overlay test (example 2): all TLC assays were carried out with HPLC-purified α -2-3- and α -2-6-sialylated neolacto series monosialogangliosides. The application is identical in all three chromatograms: lanes a: 15 μ g human brain gangliosides (HBG); lanes b: 15 μ g human granulocyte gangliosides (HGG); lanes c: 4 μ g IV³nLc4Cer (HGG1); lanes d: 4 μ g VI³nLc6Cer (HGG2); lanes e: 4 μ g IV⁶nLc4Cer (HGG3); lanes f: 8 μ g VI⁶nLc6Cer and IV⁶nLc4Cer.

[0119] **FIG. 4:** TLC test for the identification of binding motifs which are specific for ricin

[0120] (A) Orcinol staining and (B) ricin TLC overlay test with neutral GSL. The application is identical in both chromatograms. Lanes a: 10 μ g neutral GSL from human erythrocytes; lanes b: 15 μ g neutral GSL from human granulocytes; lanes c: 20 μ g neutral GSL from MDAY-D2 cells.

[0121] **FIG. 5:** TLC test with ricin on ganglioside binding

[0122] (A) Orcinol staining and (B) ricin TLC overlay test gangliosides. Lanes a: 10 μ g human brain gangliosides (HBG); lanes b: 8 μ g human granulocyte gangliosides (HGG).

[0123] **FIG. 6:** Cytotoxicity test with different sensitive cell lines

[0124] Description of the biologic activity of rViscumin: viability of HL-60 cells (points), 5637-cells (triangle) and CHO-K1 cells (open circles) were applied against the rViscumin concentration. Viability was measured by means of colorimetric reaction of WST-1 and depicted as % living cells compared to an untreated control. The half-maximal cytotoxicity which corresponds to the turning point of the curve was taken as measurable variable. These IC₅₀ values were calculated for HL-60 for 66 pg/ml and for 5637-cells for 690 pg/ml. The CHO-K1 cells are to be considered insensitive vis-à-vis rViscumin up to an applied measured rViscumin concentration of 300 ng/ml.

[0125] **FIG. 7:** Semiquantitative correlation of the sensitivity against rViscumin with the occurrence of a ganglioside band which is specific for rViscumin

[0126] (A) Orcinol staining and (B) anti IV⁶nLc4Cer antiserum TLC overlay test with gangliosides from in vitro propagated cell lines.

[0127] (A) Lane a: 7 μ g human granulocyte gangliosides (HGG); lane b: gangliosides from 1×10^7 CHO-K1 cells; lane c: gangliosides from 4×10^7 5637-cells; lane d: gangliosides from 4×10^7 HL-60 cells; lane e: 10 μ g human brain gangliosides (HBG).

[0128] (B) Lane a: 0.134 μ g human granulocytes gangliosides (HGG); lane b: gangliosides from 1×10^7 CHO-K1 cells; lane c: gangliosides from 1×10^7 5637-cells; lane d: gangliosides from 1×10^7 HL-60 cells; lane e: 10 μ g human brain gangliosides (HBG). Lane a shows both positive controls IV⁶nLc4Cer (C24 fatty acid, substance 1) and IV⁶nLc4Cer (C16 fatty acid, substance 2) for the identification of specific gangliosides of the neo-lacto series.

[0129] **FIG. 8:** Sensibilisation vis-à-vis the cell line CHO-K1 which is insensitive to rViscumin by preincubation with specific gangliosides.

[0130] Increasing amounts of human granulocyte gangliosides were placed into wells where CHO-K1 cells grow and were incubated there for 48. The cells were washed either with serum-free medium (light gray bands) or with serum-containing medium (dark bands) and were subsequently treated with 300 ng/ml rViscumin for another 48 hours. The viability was measured with WST-1 and was depicted as % to the untreated control (not with gangliosides and rViscumin).

[0131] **FIG. 9:** Enzyme-linked lectin assay (ELLA) of rViscumin with neutral GSL and gangliosides which are adsorbed to the microtiter plate

[0132] Quantities of GSL from different sources correspond to the bands from the left to the right:

[0133] A) Neutral GSL from human erythrocytes: 10, 5, 2.5, 1.25 and 0 μ g

[0134] B) Neutral GSL from human granulocytes: 15, 7.5, 3.75, 1.9 and 0 μ g

[0135] C) Neutral GSL from MDAY-D2 cells: 20, 10, 5, 2.5 and 0 μ g

[0136] D) Human brain gangliosides (HBG): 10, 5, 2.5, 1.25 and 0 μ g

[0137] E) Human granulocyte gangliosides (HGG): 10, 5, 2.5, 1.25 and 0 μ g

[0138] **FIG. 10:** Identification of specific antibodies against the ganglioside antigen used for immunisation

[0139] Test of different hybridoma clones for the production of IgM (A) and IgG (B) in ELISA after thinning out. ELISA was carried out as described in example 8. The incubation time of the substrate solution was varied (15 min,

30 min, 45 min, 60 min, 120 min and 180 min). Clones 33.3, 33.5 and 33.6 showed a clear titer on IgM (A). A titer on IgG could not be detected in any of the tested clones (B).

[0140] **FIG. 11:** Characterisation of anti- α 2-6 sialylated monoclonal ganglioside antibodies of the neolacto type

[0141] TLC overlay assay for the characterisation of the recognition motif of the mAb clones. The clones which have already been depicted in **FIG. 10** were, as described in example 2, were examined for their recognition motif in order to detect human granulocyte gangliosides (4 μ g/lane) which were separated on the DC plates. Clones 59.33.3, 59.33.5 and 59.33.6 show the binding or recognition motif identical to rViscumin.

[0142] **FIG. 12:** Further characterisation of anti- α 2-6 sialylated monoclonal ganglioside antibodies of the neolacto type

[0143] TLC overlay assays with (A) isolated neutral GSL of the neolacto series from human granulocytes (15 μ g per lane), (B) of the globo series (from human erythrocytes; 10 μ g per lane) and (C) of the ganglio series (from MDAY-D2 cells; 10 μ g per lane). The experimental proceeding is described in example 9. In the first three lanes each of **FIGS. 12A** to C mAb clones 50.33.3, 59.33.5 and 59.33.6 were used. The positive controls in lanes 4 of **FIG. 12A-C** reflect the reactions with specific antibodies against each of the terminal sugar structures of the applied neutral GSL. In lanes 5 a polyclonal antiserum from goat (cf. M \ddot{u} thing et al., *Glycobiology* 12, 485-497) is tested. A cross-reactivity of the mAb clones tested in lanes 1 to 3 can be ruled out.

[0144] **FIG. 13:** Detection of CD75s recognition of rViscumin on glycoproteins.

[0145] 1 μ g each of the corresponding proteins, in lane T: transferring, soluble protein with Neu5Aca2-6Gal β 1-4GlcNAc residues, and in lane AF: asialofetuin, (Gal β 1-4GlcNAc-residues and Gal β 1-3GalNAc-Ser/Thr) were applied on SDS gel and subsequently transferred onto a nitrocellulose membrane. In a Western blot technique it was then tried to detect the CD75s structure with rViscumin (1 μ g/ml) and subsequently with the anti-A-chains mAb TA5 and anti-mouse IgG labelled with alkaline phosphatase (cf. example 2). M reflects traces of the label. The molecular weights are indicated in kDa.

[0146] **FIG. 14:** Detection of CD75s recognition of rViscumin on glycoproteins.

[0147] 1 μ g each of the corresponding proteins, in lane T: transferring, soluble protein with Neu5Aca2-6Gal β 1-4GlcNAc residues, and in lane AF: asialofetuin, (Gal β 1-4GlcNAc-residues and Gal β 1-3GalNAc-Ser/Thr) were applied on SDS gel and subsequently transferred onto a nitrocellulose membrane. In a Western blot it was then tried to detect the CD75s structure with mAb 59.33.3 and 59.33.5 as described in example 9.

TABLE 1

Structure	Abbr.	rViscumin	Ricin
<u>Gangliosides</u>			
Neu5Ac α 2-3Gal β 1-4Glc β 1-1Cer	GM3	-	-
Gal β 1-3GalNAc β 1-4(Neu5Ac α 2-3)Gal β 1-4Glc β 1-1Cer	GM1	-	(+)
Neu5Ac α 2-3Gal β 1-3GalNAc β 1-4(Neu5Ac α 2-3)Gal β 1-4Glc β 1-1Cer	GD1a	-	-
Gal β 1-3GalNAc β 1-4(Neu5Ac α 2-8Neu5Ac α 2-3)Gal β 1-4Glc β 1-1Cer	GD1b	-	-
Neu5Ac α 2-3Gal β 1-3GalNAc β 1-4(Neu5Ac α 2-8Neu5Ac α 2-3)Gal β 1-4Glc β 1-1Cer	GT1b	-	-
Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer	IV ^s nLc4	-	-
Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer	IV ⁶ nLc4	+++++	-
Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer	VI ⁿ Lc6	-	-
Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer	VI ⁶ nLc6	+++++	-
<u>Neutral Glycosphingolipids</u>			
Gal β 1-4Glc β 1-1Cer	Lc2	(+)	+
Gal α 1-4Gal β 1-4Glc β 1-1Cer	Gb3	-	(+)
GalNAc β 1-3Gal α 1-4Gal β 1-4Glc β 1-1Cer	Gb4	-	-
GalNAc β 1-4Gal β 1-4Glc β 1-1Cer	Gg3	-	-
Gal β 1-3GalNAc β 1-4Gal β 1-4Glc β 1-1Cer	Gg4	-	+++
Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer	nLc4	-	+++++
Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer	nLc6	-	+++++
Gal β 1-4(Fuca1-3)GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer	Lewis ^x	-	-

[0148] The following examples illustrate the invention described.

EXAMPLE 1

Thin-Layer Chromatography for the Separation and Detection of Glycosphingolipids and for Specifically Detecting Them

[0149] Neutral GSL were recovered from human granulocytes, human erythrocytes and MDAY-D2 cells and gangliosides from human granulocytes and human brain (FIG. 2, FIG. 4, FIG. 5 and FIG. 7) or HPLC-purified samples were used for the analysis (FIG. 3). The corresponding samples were separated on a thin-layer chromatography plate (HPTLC plates, 10x10 cm, 0.2 mm thickness by Merck, Darmstadt # 5633) coated with silica gel. Neutral GSL were separated in solvent 1 (chloroform/methanol/water, 120/70/17 v/v/v) and gangliosides in solvent 2 (chloroform/methanol/water 120/85/20 v/v/v+2 mM CaCl₂). Neutral GSL were stained with orcinol, gangliosides were either stained with orcinol or with resorcinol (Svennerholm, 1956, 1957). Neutral GSL of human granulocytes and MDAY-D2 cells and also of gangliosides of human granulocytes appear as double bands on the DC plate.

[0150] In order to identify the individual gangliosides, an immune-staining procedure according to Müthing and Mühlradt (1988) and Müthing (1998) was carried out with specific antisera. Terminal α 2-6-sialylated gangliosides of the neolacto series were identified with a polyclonal anti-IV⁶nLc4Cer antiserum. To this avail, the plate was first fixed with polyisobutylmethacrylate (Röhm, Darmstadt). Subsequently, the plate was blocked in PBS (20 mM phosphate, 150 mM NaCl, pH 7.2)+1% BSA (solution A). In the following, the above-identified polyclonal antiserum was

used in a dilution of 1:1,000 in solution A and subsequently washed 3x with solution B (PBS+0.05% Tween 20). Bound antiserum is detected with secondary antiserum rabbit-anti-chicken-IgY in a dilution of 1:2,000 in solution A. Afterwards, it is washed 3x with solution B and 1x with glycine buffer (0.1 M glycine, 1 mM ZnCl₂, 1 mM MgCl₂, pH 10.4). The detection is carried out with 0.05% (w/v) 5-bromo-4-chloro-3-indolylphosphate in glycine buffer. The control gangliosides which were used in the different TLC tests and which are characterised in detail and which were used as reference material for the identification of the gangliosides which are specific of rViscumin originated from different isolations from human blood cells or cell cultures (Müthing et al., 1994; Müthing and Kemminer, 1996; Schwartz et al., 1985; Müthing and Cacic, 1997).

EXAMPLE 2

TLC Test for the Specific Detection of Carbohydrate-Binding Proteins, such as Ricin and rViscumin, Bound to Neutral GSL or Gangliosides

[0151] Neutral GSL or gangliosides which were separated on a thin-layer chromatography plate were washed with PBS-Tween 80 (0.1 g/L Tween 80 in PBS). Subsequently, a coating with rViscumin or ricin protein solution (1 μ g/ml in PBS-T80) was carried out and an incubation was carried out for 1 hour. Subsequently, washing was carried out with solution B (cf. example 1) and then the plate was incubated for 15 min in solution A (cf. example 1). Subsequently, the bound rViscumin was recognised with the monoclonal Ab TA5 (1 μ g/ml in solution A) described by Tonevitzky et al. 1995, or the bound ricin was recognised by a rabbit-anti-ricin antiserum by Sigma #R-1254 (1:20.0 dilution in solution A). The detection of the bound antibodies was carried

out as described in example 1 with the secondary antibodies against mouse or rabbit immunoglobulin which were needed in each case.

EXAMPLE 3

Comparison of the Sensitivity of Different Cell Lines to rViscumin

[0152] Cultivation: CHO-K1 cells (ATCC CCL-81) and HL-60 cells (ATCC CCL-240) were grown in a 1:1 mix of DMEM and Ham's F12 medium. The human bladder carcinoma cell line 5637 (ATCC HTB-9) was cultured in RPMI 1640. A cultivation of the cells was carried out under standard conditions as described in Möckel et al., 1997. The sensitivity of the cell lines to rViscumin was established in an in-vitro-cytotoxicity test. 8×10^3 5637, 1×10^4 CHO-K1 and 1.8×10^4 HL-60 each per well were plated in 96-well plates (Nunc) and incubated with increasing concentration of rViscumin in a final volume of 100 μ l for 72 h at 37° C. under a 10% CO₂ atmosphere. All measurements were carried out with sixfold determination. After 72 hours, 10 μ l WST-1 was added by a pipette in order to detect living cells (Ishiyama et al., 1993; Möckel et al., 1997). The cells were incubated with the colouring salt for 4 hours at 37° C. and subsequently measured at 450 nm in Thermomax by Molecular Devices. In this connection, the amount of dye is directly proportional to the number of living cells. In FIG. 6 the viability curves of the different cell populations are applied against increasing rViscumin concentrations.

[0153] The cell line HL-60 (points) is a human promyelocytic cell line which reacts very sensitively with an IC₅₀ value of 66 pg/ml to rViscumin. In the bladder carcinoma cell line 5637 (triangles), an IC₅₀ of 690 pg/ml was measured, the hamster cell line CHO K1. (open circles) showed no sensitivity to rViscumin up to a tested concentration of 300 ng/ml. The sensitivity of the different cell lines to rViscumin could be correlated with the occurrence of the potential receptor in FIG. 7. FIG. 7A shows an orcinol-stained thin-layer chromatogram of the complete ganglioside fractions of the cell lines used in each case. FIG. 7B shows the specific immunologic detection of the potential rViscumin receptors with an anti-Neu5Ac α 2-6Gal β 1-4GlcNAc-R antiserum (overlay assay). In lanes b, c and d of FIG. 7B, ganglioside amounts of identical cell numbers of CHO-K1, 5637 and HL-60 cells, respectively, are applied. The CHO-K1 cells, which are insensitive to rViscumin show no specific band on the level of the potential receptor in lane b of FIG. 7B, while, with the same number of cells applied, the most sensitive cell line (HL-60) shows the strongest double band. From the different specificities of the lectins examined which were observed in the TLC overlay assays conclusions can be drawn on the binding behaviour of ricin and mistletoe lectin (particularly rViscumin) to their receptors in vivo or in cell cultures. The examples describe that the CHO-K1 cell line is insensitive to a tested rViscumin concentration of up to 300 ng/ml (cf. FIG. 6). By introducing the gangliosides which are specific for rViscumin into the membranes of the CHO-K1 cells (Neu5Ac α 2-6 Gal structures), these cells which are very insensitive to rViscumin were, however, sensibilised (cf. FIG. 8).

EXAMPLE 4

Sensibilisation of Cell Lines by Preincubation with Specific Gangliosides (cf. FIG. 8)

[0154] CHO-K1 cells were sown with a cell density of 5×10^4 cells per well in 96-well plates. The cells were cultivated for 24 hours in a serum-free X-Vivo culture medium (Biowhittaker) at 37° C. Subsequently, the medium was discarded and was replaced by X-Vivo culture medium containing 0 (control), 25, 50, 100 and 200 μ M of a human granulocyte ganglioside fraction. In order to incorporate the gangliosides into the membrane of the CHO-K1 cells, the cells were kept for 48 hours at 37° C. under cell culture conditions. Subsequently, the cells were either extensively washed with serum-free X-Vivo or with 5% FCS-containing X-Vivo. Then, the cells were treated with rViscumin (300 ng/ml) for 48 hours. The viability of the cells was quantified with WST-1 staining and were correlated with the untreated control (without addition of rViscumin and gangliosides). The samples were grown on in triplicates with an end volume of 100 μ l per well.

EXAMPLE 5

Purification of Gangliosides of In-Vitro Propagated Cells and Correlation of the Occurrence of Specific Gangliosides in Cell Lines and Sensitivity to rViscumin

[0155] Showing the cell mass for the subsequent isolation of GSL or gangliosides: CHO-K1 cells (ATCC CCL-81) and HL-60 cells (ATCC CCL-240) were grown in a 1:1 mix of DMEM and Ham's F12 medium. The cells were cultivated in the presence of 50 mg/L gentamycin and 2.5 mg/L amphotericin B. A cultivation of 5637-cells was carried out in 175 cm²-flasks. HL-60 and CHO-K1 cells were cultivated in a 1 L-Spinner flask as described in Duvar et al. (1996) and Muthing et al (1996b). The physiological parameters were: 37° C., pH 7.2, fumigation with oxygen, stirring velocity: 40 rpm. The cells were harvested after reaching the final cell densities, washed twice with PBS and subsequently subjected to a chloroform/methanol extraction (cf. example 1). The human bladder carcinoma cell line 5637 (ATCC HTB-9) was cultivated in RPMI 1640. The gangliosides were isolated on DEAE sepharose CL-6B with standard procedures using anion exchanger chromatography, as described by Ledeen and Yu (1982), Müthing et al. (1987) or Duvar et al (1997). For saponification of the phospholipids, the gangliosides were incubated for 1 hour at 37° C. in 1 N NaOH. Subsequently, the ganglioside fraction was neutralised with acetic acid and dialysed. A further purification step followed by adsorption chromatography to latrobeads 6RS-8060 (Macherey & Nagel, Düren, Germany) (Ueno et al., 1987). When the ganglioside fraction was subsequently separated from the cell lysates, it was always assumed that the number of cells was always approximately the same. Thus, it was possible to make a semiquantitative statement with respect to the presence of the specific ganglioside. The detection was carried out immunologically as described in example 1 with a polyclonal anti-IVⁿLc4Cer antiserum.

EXAMPLE 6

Purification of Terminal α 2-3- and α 2-6-Sialated Gangliosides of the Neolacto Series

[0156] A column filled with Fractogel EMD TMAE-650-S (MERCK, Darmstadt) is filled with the gangliosides from

human granulocytes (HGG). TMAE-bound gangliosides are eluted with a linear gradient to ammoniumacetate, pooled and deionized. Four fractions with terminal α 2-3-sialated (HGG1 and HGG2) and terminal α 2-3-sialated neolacto type monosialogangliosides (HGG3 and HGG4) were obtained. All gangliosides were separated on thin-layer chromatograms as double band. The differences are due to the ceramide unit, which, in the case of the upper band, esterified a C24 fatty acid and, in the case of the lower band, a C16 fatty acid. The structures of all gangliosides which can be depicted with the methods have already been characterised (Müthing et al., 1993; Müthing et al., 1996; Metelmann et al., 2000). The purified gangliosides have been separated in **FIG. 3** and were referred to for the closer specification of the carbohydrate-binding specificity of rViscumin.

EXAMPLE 7

Enzyme-Linked Lectin Assay (ELLA)

[0157] The ELLA was coated in plates which are coated with the corresponding neutral GSL or gangliosides (NUNC MaxiSorp F96, Wiesbaden) at room temperature. The wells were filled with the corresponding GSL in 100 μ l methanol. Starting from a GSL stock solution, 1:2 dilutions were prepared. This contains the following GSL: neutral GSL of human erythrocytes: 10, 5, 2.5 and 1.25 μ g; neutral GSL of human granulocytes: 15, 7.5, 3.75 and 1.9 μ g; neutral GSL from MDAY-D2 cells: 20, 10, 5 and 2.5 μ g; human brain gangliosides (HBG): 10, 5, 2.5 and 1.25 μ g; human granulocyte gangliosides (HGG): 10, 5, 2.5 and 1.25 μ g. Subsequently, the methanol was evaporated in a dry atmosphere (45 min at 37° C.). All further steps were carried out as in the TLC overlay assay described in example 2. All incubations were carried out in a volume of 100 μ l. The GSL-coated plates were incubated for 15 min with PBS-Tween 80® (0.01 mg/ml) and subsequently incubated with 1 μ g rViscumin (dissolved in PBS-Tween 80) for 1 hour. Afterwards, the plates were washed with PBS-Tween 20® (0.01 mg/ml) and then incubated with PBS for 15 minutes. Subsequently, the samples were incubated with murine anti-ML-1 A monoclonal antibody TA5 (1 μ g/ml in PBS) for 1 hour. After again washing for three times with PBS-Tween 20®, an incubation in PBS with an anti-mouse IgG (alkaline phosphatases coupled) in a 1:2000 dilution was carried out for 1 hour. After again washing for three times with PBS-Tween 20®, a colorimetric detection is carried out with a reaction of the substrate disodium-4-nitrophenylphosphatex 6H₂O (16 mM in 0.1 M glycine buffer, pH 10.4). The plate was measured after an incubation for 20 minutes at 25° C. at 405 nm in a microtiter plate reader.

[0158] Unexpectedly, a binding of rViscumin could only be found to such glycosphingolipids (GSL) which terminally exposed an α 2-6-bound galactose to GSL of the neolacto series at the Neu5Ac (HGG fraction from human granulocytes). All other neutral GSL from different sources (human granulocytes and erythrocytes, MDAY-D2 cells), each with terminal galactose residues and human brain gangliosides of the ganglio series showed no binding even in the highest concentration used.

EXAMPLE 8

Production of Monoclonal Antibodies Against α 2-6-Sialylated Gangliosides of the Neolacto Type

[0159] The α 2-6-sialylated gangliosides of the neolacto type were isolated from leucocytes (buffy coat from whole

blood). The purification of the gangliosides was carried out as described in example 5. In the further process, these α 2-6-sialylated gangliosides of the neolacto type served as antigens for the immunisation of 2x3 mice. As the antigens have no immunogenic effect alone due to their lack in size, they are administered to the mice with so-called haptens. In this connection, two methods were carried out in parallel. In method 1 methyl-BSA was used as hapten in a concentration of 1 mg/ml. The three mice received different amounts of the antigen (5, 10 and 20 micrograms) The mice were immunised in known intervals and received a booster altogether two further times with an interval of 10 days. In method 2 a mixture of liposomes and lipopolysaccharide (LPS) was used as hapten. Here, too, different amounts of the antigen (5, 10 and 20 micrograms) were administered to the mice on three application days, each, with an interval of 10 days.

[0160] From the altogether six mice which were immunised with the help of two methods, a positive titer against the antigen could be detected in the blood of three of the mice. The preparations with the hapten methyl-BSA were negative. The preparations with the hapten lipopolysaccharide in liposomes were all positive, independent of the dose of the antigen administered. A mouse was sacrificed and the spleen cells were used for fusion. The supernatants of the fusion assays were examined in the DC-overlay assay (as described in examples 1 and 2) and in ELISA. In this process the microtiter plate was coated with 1 μ g HGG gangliosides (human granulocyte gangliosides) in 50 μ l methanol as described in example 7. 50 μ l of the corresponding plasma samples or supernatants of the fusion assays were applied undiluted to the coated plates and were incubated for 60 minutes at 25° C. Subsequently, washing was carried out 3 times with PBS-T. A detection of IgM or IgG was carried out with an anti-mouse-IgG or an anti-mouse-IgM antibody which was labelled with alkaline phosphatase. A reaction of the substrate p-NPP was carried out as described in example 7. Fusion assays with a positive titer were subsequently thinned out on the clone level. **FIG. 10A** shows a detection for three positive IgM clones (59.33.3, 59.33.5 and 59.33.6). All clones were also examined for the production of IgG, however, they showed no positive reaction to IgG (**FIG. 10B**).

EXAMPLE 9

Characterisation of anti- α 2-6-Sialylated Monoclonal Ganglioside Antibodies of the Neolacto Type

[0161] α 2-6-sialylated gangliosides of the neolacto type always carry a Neu5Ac α 2-6-Gal at their end. Another motif, which was also described in the course of this invention, comprises the ganglioside structure Neu5Ac α 2-6-Gal β 1-4GlcNAc-R; wherein "R" represents a residue. This motif has been described in the literature as CD75s (Schwartz-Albiez (2000), J. Biol. Regul. Homeost. Agents 14, 284-285). **FIG. 11** shows DC-overlay assays with various IgM clones which were tested in ELISA as described in example 8. Here, the gangliosides from human granulocytes (HGG) were separated with the help of thin-layer chromatography. The assay was carried out as described in example 2. Here, 4 μ g HGG were separated. The detection of anti-CD75s mAb bound to the plate was carried out as described in example 8. Clones 59.33.3, 59.33.5 and 59.33.6 showed a

positive signal in this test, too. All three clones recognised the same gangliosides (IV₆nLC4 and IV₆nLC6) and, thus, had a binding behaviour identical to rViscumin (**FIG. 2Bb**). A cross-reactivity of the three antibodies against the core part of the sugar structures, which can possibly be expected, could be ruled out experimentally by using isolated neutral GSL from either the neolacto series from human granulocytes (15 µg per lane; **FIG. 12A**) or the globo series (from human erythrocytes, 10 µg per lane in **FIG. 12B**) and the ganglio series (from MDAY-D2 cells; 10 µg per lane; in **FIG. 12C**). The experimental approach was identical to the one described above. In the first three lanes, each, of **FIG. 12A** to **C**, no signals could be detected by using the mAb clones 59.33.3, 59.33.5 and 59.33.6. In all three cases did the absence of the terminal α2-6-linked sialic acid lead to the absence of a specific signal. The positive controls in lanes 4 of **FIG. 12A** to **C** reflect the reactions with specific antibodies against the respective terminal sugar structures of the neutral GSL applied. In lanes 5 a polyclonal antiserum from goat (cf. Mühling (2002) *Glycobiology* 12, 485497) was carried along. This antiserum which was originally prepared against α2-6-sialylated gangliosides of the neolacto type shows a cross-reactivity against many of the antigens which were applied here, which could lead to false positive signals, particularly when using this antiserum in the examination of material from patients. The results with the monoclonal antibodies 59.33.3, 59.33.5 and 59.33.6, shown in lanes 1 to 3, where such undesired cross-reactivities did not occur, have to be considered more important. The mAb-clone

59.33.3 was deposited with the DSMZ in Brunswick on 20 Dec. 2002 under the accession number [. . . number] according to the Budapest Treaty.

EXAMPLE 10

Detection of CD75s on Glycoproteins

[0162] The monoclonal antibodies 59.33.3 and 59.33.5 (**FIG. 14**) and rViscumin (**FIG. 13**) themselves were examined for their usability for the detection of CD75s motifs on glycoproteins. Here, 1 µg, each, of the corresponding proteins, in lane T: transferrin, soluble protein with NeuSaca2-6Galβ1-4GlcNAc-residues and in lane AF: asialofetuin, (Galβ1-4GlcNAc residues and Galβ1-3GalNAc-Ser/Thr) were applied to the SDS gel and subsequently transferred to a nitrocellulose. In a Western blot it was then tried to detect the CD75s structure either with rViscumin (1 µg/ml) and subsequently with the anti-A-chain mAb TA5 and with the anti-mouse-IgG labelled with alkaline phosphatase (cf. example 2). In the case of the examination of the bond/recognition of the CD75s epitope by the mAb 59.33.3 and 59.33.5, after the transfer of the proteins to the nitrocellulose, the same was done as described in example 9. The transferrin is unambiguously recognised by the mAbs 59.33.3 and 59.33.5 and by rViscumin.

[0163] Both antibody clones 59.33.3 and 59.33.5 tested recognise CD75s motifs on the glycoprotein transferrin as epitopes.

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 645

1. An in vitro method for the determination of the responsiveness of an individual to mistletoe lectin or to one or more mistletoe lectin single chains, comprising quantitative and/or qualitative detection of a membrane-bound receptor, wherein the receptor is characterized by a terminal N-acetyl neuraminic acid (Neu5Ac) which is linked to a galactose (Gal) by a glycosidic α 2-6 bond.

2. The method according to claim 1, wherein the receptor comprises a ganglioside which after the N-acetyl neuraminic acid bound to galactose via an α 2-6 bond comprises an N-acetyl glucosamine (GlcNAc).

3. The method according to claim 1, wherein the receptor comprises a ganglioside of the structure Neu5Ac α 2-6-Gal-[Gal/GlcNAc]_k-Cer.

4. The method of claim 1 any one of claims 1 to 3, wherein the receptor comprises a ganglioside with the structure Neu5Ac α 2-6-[Gal β 1-4GlcNAc β 1-3]_xGal β 1-4Glc β 1-1Cer.

5. The method of claim 1, wherein the receptor comprises a ganglioside with the structure Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer or a ganglioside with the structure Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer.

6. The method of claim 1, wherein the receptor is cell membrane-bound.

7. The method of claim 1, wherein the mistletoe lectin is a recombinant mistletoe lectin/rViscumin.

8. The method of claim 7, wherein the recombinant mistletoe lectin comprises an amino acid sequence which is encoded by a polynucleotide as shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO:5.

9. The method according to claim 7, wherein the recombinant mistletoe lectin is a polypeptide with an amino acid sequence as shown in SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6 or comprises a functional fragment thereof.

10. An in vitro method for the determination of a responsiveness to mistletoe lectin(s), comprising quantitative and/or qualitative determination of at least one α 2,6-sialyltransferase.

11. A diagnostic composition comprising a substance which specifically recognizes or binds to a receptor as defined in claim 1 and is selected from the group consisting of antibodies, antibody derivatives, antibody fragments, aptamers, low-molecular substances and carbohydrate-binding peptides.

12. The diagnostic composition according to claim 11, wherein the substance is detectably labelled.

13. The diagnostic composition according to claim 11, wherein the antibody is a monoclonal antibody.

14. The diagnostic composition according to claim 13, wherein the monoclonal antibody can be recovered from a hybridoma cell line which was deposited on 20 Dec. 2002 with the DSMZ Braunschweig under the accession number DSM ACC2580.

15. A method of detecting a functional mistletoe lectin receptor, comprising contacting one or more cells with the diagnostic composition of claim 11.

16. The method according to claim 15, wherein the diagnostic composition detects a membrane-bound receptor on one or more cells, wherein the receptor is characterized by a terminal N-acetyl neuraminic acid (Neu5Ac) which is linked to a galactose (Gal) by a glycosidic α 2-6 bond.

17. The method according to claim 16, wherein the one or more cells are tumor cells.

18. The method according to claim 16, wherein the one or more cells originate from biopsy material.

19. The method according to claim 16, wherein the one or more cells are isolated from blood samples.

20. A method of treating a proliferative disease, viral disease, autoimmune disease or neuronal disease, comprising administering a pharmaceutical composition to an individual, wherein the pharmaceutical composition comprises a substance that specifically binds to a receptor or recognizes a membrane-bound receptor, wherein the receptor is characterized by a terminal N-acetyl neuraminic acid (Neu5Ac) which is linked to a galactose (Gal) by a glycosidic α 2-6 bond and wherein the receptor-binding or receptor-recognizing substance is selected from the group consisting of antibodies, antibody derivatives, antibody fragments, aptamers, low-molecular substances and carbohydrate-binding peptides.

21. The method according to claim 20, wherein the receptor-binding and/or receptor-recognizing substance is linked to a compound having a radioactive, a cytotoxic or a cytostatic effect.

22. The method according to claim 21, wherein the compound linked to the receptor-binding and/or receptor-recognizing substance is a peptide.

23. The method according to claim 20, wherein the antibody is a monoclonal antibody.

24. The method according to claim 23, wherein the monoclonal antibody can be recovered from a hybridoma cell line deposited on 20 Dec. 2002 at the DSMZ Braunschweig under the accession number DSM ACC2580.

25. The method according to claim 20, wherein the receptor-binding and/or receptor-recognizing substance further comprises a domain which can induce an immunologic effector function.

26. The method according to claim 25, wherein the induced immunologic effector function is a cellular effector function.

27. The method of claim 26, wherein the induced immunologic effector function is a humoral effector function.

* * * * *

专利名称(译)	确定个体对槲寄生凝集素的反应性的方法		
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申请号	US10/499297	申请日	2002-12-20
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[标]发明人	MUTHING JOHANNES PETER KATALINIC JASNA LANGER MARTIN MOCKEL BABETTE ECK JURGEN		
发明人	MUTHING, JOHANNES PETER-KATALINIC, JASNA LANGER, MARTIN MOCKEL, BABETTE ECK, JURGEN		
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摘要(译)

一种测定个体对槲寄生凝集素或 (a) 槲寄生凝集素单链的反应性的方法，其中槲寄生凝集素的膜结合受体的表达是相应反应性的特征。

Figure 1

