LECTIN-ENZYME BINDING ASSAYS

Development of the technique and applications in biochemistry and medicine

LECTINE-ENZYM BINDINGS ANALYSES

Ontwikkeling van de techniek en enige toepassingen in de biochemie en de geneeskunde

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CONTENTS

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1. Pekelharing JM, Vissers P, Peters HA, Leijnse B. Lectin-enzyme immunoassay of transferrin sialovariants using immobilized antitransferrin and enzyme labeled galactose-binding lectin from Ricinus communis. Anal Biochem 1987, 165: 320-326.
2. Pekelharing JM, Hepp E, Kamerling JP, Gerwig GJ, Leijnse B. Alterations in carbohydrate composition of serum IgG from patients with rheumatoid arthritis and from pregnant women. Annals Rheum Diseases 1988, 47: 91-95.
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4. Pekelharing JM, Mos P. The determination of equivalency between lectin-biotin and streptavidin-peroxidase without using the lectin binding function. (submitted).
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6. Pekelharing JM, Mos P. The binding of lectin-streptavidin-peroxidase complexes to purified human plasma glycoproteins adsorbed in microtiter plate wells. (submitted).
7. Pekelharing JM, Peters HA. The inhibition by simple sugars of the binding of enzyme-labelled lectin from Ricinus communis to asialotransferrin adsorbed in microtiter plate wells. (submitted).
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LIST OF ABBREVIATIONS

AAA	Anguila anguila agglutinin
AAL	Aleuria aurantia lectin
ACA	Artocarpus communis agglutinin
AIA	Artocarpus integrifolia agglutinin ("Jacalin")
APA	Abrus precatorius agglutinin
βG III	β_2-glycoprotein III
BPA	Bauhinia purpurea agglutinin
BSII	Bandeiraea simplicifolia agglutinin
CJA	Crotalaria juncea lectin
Con A	Concanavalin A agglutinin
DSA	Datura stramonium agglutinin
ECA	Erythrina cristagalli agglutinin
ECO	Erythrina corallodendrum agglutinin
EEA	Euonymus europaeus agglutinin
$F(ab')_2$	Antibody-binding fragment of IgG prepared with pepsin
FSH	Follicle stimulating hormone
Fuc	Fucose
Gal	Galactose
GalNAc	N-Acetyl-galactosamine
GlcNAc	N-Acetyl-glucosamine
Gc	Vitamin D binding glycoprotein
GnT	β-N-acetylglucosaminyl transferase
HCG	Human chorionic gonadotropin
IAP	Immunosuppressive acidic protein
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Ka	Association constant
LCA	Lens culinaris agglutinin
LH	Luteinizing hormone
LPA	Limulus polyphemus agglutinin
Man	Mannose
MPA	Maclura pomifera agglutinin
NeuNAc	N-Acetyl-Neuraminic Acid
PBS	Phosphate-buffered saline
PCA	Phylodendron californicum agglutinin
PHA	Phaseolus vulgaris agglutinin ("phytohemagglutinin")
PNA	Peanut agglutinin (Arachis hypogaea)
PSA	Pisum sativum agglutinin

RA	Rheumatoid arthritis
RCA	Ricinus communis agglutinin I
RF	Rheumatoid factor
SLE	Systemic lupus erythematodes
STA	Solanum tuberosum agglutinin
STAP	Streptavidin-biotinylated-peroxidase complex
TBS	Tris-buffered saline
TF	Transferrin
TMA	Tridacna maxima agglutinin
TSH	Thyroid-stimulating hormone
UEA	Ulex europaeus agglutinin
VUA	Vigna unguiculata agglutinin
VVA	Vicia vilosa agglutinin
WGA	Wheat germ agglutinin (Triticum vulgaris)

CHAPTER 1

LECTINS AND THE INVESTIGATION OF PLASMA PROTEIN GLYCOSYLATION

1.1. Introduction

Glycoproteins consist of a protein part and a carbohydrate (or glycosylated) part. The latter may be investigated by using carbohydrate binding proteins called lectins. This chapter describes, in detail, aspects of protein glycosylation and its study using lectins. The feasability of a new approach is also discussed. This would involve using lectins to replace the immobilised or enzyme-linked antibody in sandwich ELISA techniques.

1.2. Lectins

1.2.1. *History (compiled from Liener et al., 1986)*

Exactly one hundred years ago Stillmark (1888) observed the hemagglutination of red blood cells by a solution from ground castor beans. His work started a series of papers and theses from Kobert's laboratory at the University of Dorpat, Estonia (now Tartu, USSR) on agglutinating toxins. Erythrocytes from different animals reacted differently. Other seeds and plants were found to possess similar, but not identical properties. Lectinology was born.

Subsequent experiments established the protein nature of the lectins, and their agglutinating, as well as precipitating activities, their varying selectivity of interaction with different cells, and the inhibition of their activity by certain substances. Eminent investigators such as Paul Ehrlich, the father of modern immunology and Karl Landsteiner, discoverer of the ABO and Rhesus blood groups, studied plant agglutinins in the early period. The use of certain lectins as blood group specific reagents initiated a new wave of interest in lectins that resulted in an avalanche of papers in the first half of the century, many of them dealing with systematic screening of thousands of plant species. Another important finding was the mitogenic properties of lectins of Phaseolus vulgaris seeds, inappropriately named "phytohemagglutinin" or PHA.

Lectins were found to be widely distributed in nature: seeds, plants, fungi, bacteria, viruses, invertebrates and vertebrates all contained soluble or membrane-bound lectins. Some proved to be extremely toxic, the compounds responsible for the toxicity were later termed toxins.

Watkins and Morgan were the first to show that simple sugars are capable of inhibiting lectin activity. These studies helped to resolve the carbohydrate structures of the blood group determinants (Watkins, 1980).

Terminology has changed to some extent during the first hundred years. The oldest names of lectins were derived form the plant source: ricin from Ricinus communis, crotin from Croton tiglium, robin from Robinia pseudoacacia, abrin from Abrus precatorius, and many others. Later collective names were suggested such as "phasins", "hemagglutinins", "phytagglutins", and "prolectins". The word "phytohemagglutinin" and its abbreviation PHA is still used for the lectins from the red kidney bean Phaseolus vulgaris.

The term "lectin" was introduced by Boyd in 1954: "It would appear to be a matter of semantics as to whether a substance not produced in response to an antigen should be called an antibody even though it is a protein and combines specifically with certain antigens only. It might be better to have a different word for these substances and the present writer would like to propose the word "lectin", from the Latin "lectus" the past principle of "legere" meaning to pick, choose or select" (Boyd et al., 1954).

With increasing knowledge of the nature of lectins it became evident that most of them were carbohydrate specific. During the second half of the 1960s "lectin" gradually replaced the term "hemagglutinin" and some agglutinins of animal and bacterial origin were also classified as lectins. On the other hand, with most of the viral hemagglutinins the name lectin has been used with some hesitation up to the present.

1.2.2. *Definitions*

Lectins are usually defined as carbohydrate-binding proteins of non-immune origin that agglutinate cells or precipitate polysaccharides or glycoconjugates (Goldstein et al., 1980). This definition has been adopted by the Nomenclature Committee of the International Union of Biochemistry IUB (Dixon, 1981). The emphasis on "non-immune" origin distinguishes lectins from anti-carbohydrate antibodies, such as anti-blood group antibodies which may also act as cell agglutinins.

The definition is an operational one which excludes monovalent proteins and other sugar-binding proteins such as glycosidases and glycosyltransferases. The border between enzymes and lectins is still unclear in a number of instances where proteins seem to be able to act as both (Hankins et al., 1978; Kelleher et al., 1988). An example is the copper containing enzyme galactose oxidase, which is also a hemagglutinin, especially at lower temperatures where the enzyme function is diminished. In its apo form with copper removed the enzymatic activity is lost, but the protein fully retains its lectin function (Horejsi et al., 1979).

1.2.3 *Occurrence*

Although known as plant and seed proteins for exactly a century, it is only during the last decade that it has become clear that lectins occur in all living matter. In human beings, for instance, the first lectin to be discovered was the hepatic galactose receptor or hepatic binding protein (Ashwell et al., 1974). The paper became a "citation classic" (Current Contents 1986, 29: 18). Subsequently lectins were found in nearly all cell types and tissues and even in plasma. Well-known human proteins such as Laminin, Thrombospondin, CRP, Glycophorin, Amyloid P component, C1q, Interleukin-1, Interleukin-2, Conglutinin, Tumor Necrosis Factor and von Willebrand's factor have lectin properties (Ikeda et al., 1987; Summerfield et al., 1986; Kawasaki et al., 1986; Matsumoto et al., 1986; Lieberman et al., 1984; Hamakazi, 1986, 1988; Li et al., 1984; Taylor et al., 1984, 1987; Kataoka et al., 1985; Bowles et al., 1977; Uhlenbruck et al., 1979; Hirabayashi et al., 1984; Roberts et al., 1986; Oka et al., 1988; Baatrup, 1987; Thiel et al., 1987; Sherblom et al., 1988). Lectins can be looked upon as the "immunoglobulins" of invertebrates (Renwrantz, 1983), although no inductive synthesis stimuli are apparent, and their occurrence on vertebrate cell membranes may represent an evolutionary ancient binding mechanism (Simpson et al., 1978).

The striking amino acid sequence homology of the human lymphocyte receptor for IgE and the chicken liver asialoglycoprotein receptor (hepatic lectin) suggests the existence of a membrane lectin /receptor superfamily (Kikutani et al., 1986; Matsunaga et al., 1987). The puzzling relationship between the receptor for insulin-like growth factor II (IGF-II) and the lysosomal mannose-6-phosphate receptor suggests the same (Morgan et al.,1987; Roth, 1988).

Membrane-associated lectins are thought to function in the clearance of glycoproteins and cells from the circulation, in intracellular routing of glycoproteins and as recognition determinants between cells. Bacterial surface lectins mediate the sugar-specific adherence of bacteria to epithelial cells, which is an essential prerequisite for infection. In plants, they may serve as packaging aids for other proteins in the so-called protein bodies (Freier et al., 1987).

1.2.4. *Specificity*

Originally it was believed that the specificity of a lectin could be described in terms of the monosaccharides that best inhibited lectin-induced agglutination of animal cells. Mäkelä (1957) suggested that lectin-reactive monosaccharides could be divided into four classes based on their configuration at C-3 and C-4 of the pyranose ring. As yet, no lectin has been discovered that interacts with Mäkelä's group IV sugars (idose, gulose, L-glucose, L-xylose).

Many techniques have been described to determine the carbohydrate specificity of lectins. They include:

- precipitation reactions
- hemagglutination reactions
- equilibrium dialysis
- UV-spectroscopy
- fluorescence spectroscopy
- solid-phase adsorption-elution
- lectin column chromatography.

The present work describes lectin-enzyme binding assays.

In the later years it has become clear that most lectins bind to carbohydrate structures extending to three or more monosaccharides (Kabat, 1978). They allow some variation at C-2 of the sugars that they bind, but in general they tolerate very little variation at C-3. The C-4 hydroxyl group is often also critically involved in lectin binding. Besides, hydrophobic interaction with protein structures may add to the lectin binding.

1.2.5. *Lectin methods*

In recent years methods have been developed to study protein glycosylation. Table 1-1 lists a number of methods that have been described.

Table 1-1. Some methods used to study protein glycosylation

Lectin column chromatography
Lectin affino-diffusion
Crossed affino-immuno-electrophoresis
Isoelectric focusing
Chromatofocusing
SDS-PAGE followed by lectin-autoradiography,
lectino-fixation,
lectin-enzyme blotting
Radio-lectin immunoassay
Enzyme-linked lectin binding assay
Purification, followed by GLC, NMR or MS

To describe the exact composition of the glycans of plasma glycoproteins, purification of the glycoprotein is necessary, followed by removal and characterisation of the carbohydrates. However, methods have been developed to get an impression of the constitution of the glycan chains of the protein while it is still present in a mixture or in crude form. These methods use lectins as an affinity adsorbent in affinity electrophoresis, crossed affinity immuno-electrophoresis (crossed "affino-electrophoresis") and lectin column chromatography. These methods are very time consuming, and do not allow the simultaneous binding study of a number of samples with a number of lectins, although the latter allows the study of several glycoproteins (Jackson et al., 1988).

In conclusion, until recently no sensitive and fast techniques have been developed to characterise the glycan chains of a glycoprotein present in a complex mixture such as plasma. It is the subject of the present work to develop enzyme-linked binding assays using lectins to investigate glycoprotein glycosylation.

1.3. **Protein glycosylation**

Concentrations of plasma glycoproteins may change in disease. These changes may provide diagnostic information, which will not be discussed here. Independently, a number of co-translational or post-translational modifications in the structure of glycoproteins may also be found (Rucker et al., 1988; Wold, 1981). Tyrosine sulfation for instance, occurs in trans-Golgi (Baeuerle et al., 1987). Incomplete precursor peptide cleavage has been observed in liver disease (Peters, 1986, 1987). Sulfhydryl crosslinking takes places (Lernmark et al., 1987). The non-enzymatic addition of glucose as a fructosamine group to plasma proteins and hemoglobin is well established in patients with diabetes (Armbruster, 1987). Tests have been developed to quantitate these products. The γ-carboxylation of some clotting factors may also be modified as the result of nutritional or liver disorders (Soulier et al., 1986). And last but not least, modification of the enzymatic plasma protein glycosylation may occur.

Glycosylation occurs in the Golgi system (Kornfeld et al., 1985). Two main structures have been discovered: one has a linkage of a Man(Man-) Man - GlcNAc - GlcNAc - unit to the amide nitrogen of asparagine of an Asn-X-Ser (Thr) sequence on the glycoprotein. The N-linked glycosylation steps are shown in figure 1-1. The other is the linkage of a -GalNAc- unit to the oxygen of serine or threonine. Examples of the two basic structures are given in figure 1-2.

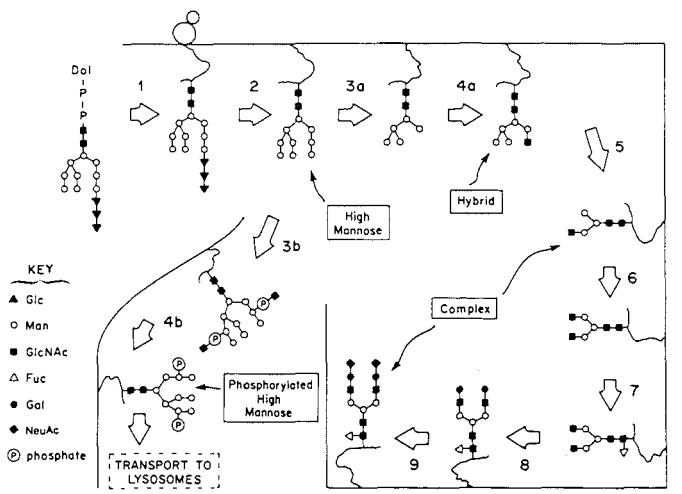
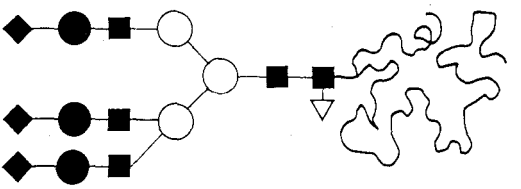


Figure 1-1. Intracellular synthesis of N-linked glycan chains (from Berger et al., 1982, with permission).

NeuNAcα2-6Galβ1-4GlcNAcβ1-2Manα1
\6
Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn
/3
NeuNAcα2-6Galβ1-4GlcNAcβ1-2Manα1
NeuNAcα2-3Galβ1-3GalNAcα1-Ser(Thr)

Figure 1-2. The basic N- and O-linked glycan structures.

Most proteins present in human plasma carry one or more N-linked carbohydrate side chains. A few also have O-linked chains (Uhlenbruck et al, 1979). As table 1-2 shows, only albumin and a few minor proteins are not glycosylated.



GLYCO	PROTEIN
Carbohydrate part has influence on:	Protein part determines function:
- half-life	- hormone
- solubility	- clotting factor
- "homing"	- transport protein
- activity of protein part	- immunoglobulin
- conformation in solution	- complement component
- resistance to proteolysis	- enzyme inhibitor
- antigenicity	- other
- cellular uptake and processing	
- postsynthesis intracellular routing and organelle localisation	
- heat stability	
- etc.	

Text-figure 1-1. Functions attributed to protein part and carbohydrate part of glycoproteins.

Table 1-2. Plasma concentration and carbohydrate content of a number of plasma glycoproteins.

Protein	Plasma concentration (g/l)	Carbohydrate content (%)
Prealbumin	0.25	0
Albumin	40	0
α_1-acid glycoprotein	1	45
α_1-lipoprotein	3	2
α_1-antitrypsin	3	12
α_2-macroglobulin	3	8
Haptoglobin	2	16
Ceruloplasmin	0.2	7
Fibronectin	0.3	3
Hemopexin	0.9	23
Transferrin	2	5
β-lipoprotein	3	2
Complement C3	1	3
Fibrinogen	2.5	4
IgG	12	3
IgA	2.1	8
IgM	1.4	10
CRP	0.01	0

1.3.1. *Roles of glycosylation*

A number of characteristics that have been attributed to the glycan structure is given in text-figure 1-1. However, large differences between individual glycoproteins have been noted. The role of a certain glycan structure in one glycoprotein may not be the same in another glycoprotein. On the other hand, certain characteristics appear to be generalized. It has been found that glycan structures may carry information that could be clinically useful. For instance, organ-specific glycosylation occurs which allows in theory the determination of the contribution of an organ to the plasma concentration of a glycoprotein (Parekh et al., 1987; Yamashita et al., 1983). The determination of the bone isoenzyme of alkaline phosphatase by affino-electrophoresis in wheat germ agglutinin containing gels is the first application in the clinical setting of a separation method based on organ-specific glycosylation (Rosalki et al., 1986; Behr et al., 1986; Onica et al., 1987). Possible roles of the glycan chains have been reviewed recently (Olden et al., 1985; West, 1986; Baenziger, 1985; Jourdian et al., 1981; Hatton et al., 1983; Schachter, 1984; Martinez et al., 1987; Bürgi, 1988; Rademacher et al., 1988).

Table 1-3 lists a number of recent representative reports describing correlations between certain cellular or biochemical characteristics and alterations in glycosylation pattern.

Table 1-3. Cellular and biochemical processes in which glycosylation plays a role.

Process	Reference
Growth	(Ceccarini et al., 1975)
Differentiation	(Katoaka et al., 1985)
Migration	(Lin et al., 1985)
Stimulation	(Yohe et al., 1985)
Viral genome expression	(McClure et al., 1983)
Leukemic transformation	(Westrick et al., 1983)
Metastatic pattern	(Varani et al., 1983)
Phagocytosis	(Sharon, 1984) Nk-cell
cytotoxicity	(Pospisil et al., 1986)
Suppressor T-cell activity	(Tosato et al., 1983)
Cell localisation	(Madyastha et al., 1980)
Homing	(Hooghe et al., 1984)
Adhesion	(Rauvala et al., 1983)
Embryonic development	(Lennarz, 1983)
Aplastic anemia	(Yoshida et al., 1987)
Sickle cell anemia	(Riggs et al., 1977)
Thalassemia	(Rachmilewitz et al., 1980)
ESR	(Levinsky et al., 1980)
Complement lysis	(Rademaker et al., 1981)
Plasmodium falciparum invasion	(Cartron et al., 1983)
Mycoplasma pneumoniae attachment	(Loomes et al., 1984)
Hypertension	(Reznikova et al., 1984)
Senescent cell removal	(Schlepper-Schdfer et al, 1983)
HLA recognition	(Pimlott et al., 1986)
IgE synthesis	(Huff et al., 1986)
B-cell suppressor factor	(Fleisher et al., 1981)
Regeneration	(Enrich et al., 1988)
T cell: B cell interaction	(Kearse et al., 1988)
Enzyme activation energy	(Delanghe et al, 1988)
Ageing	(Nakamura et al., 1988)
Inflammation	(Hachulla et al., 1988)
Tumorigenicity	(Pettijohn et al., 1988)
Hormone receptor binding	(Ulloa-Aguirre et al., 1988)
Hormone biological activity	(Ulloa-Aguirre et al., 1988)
Plasma protein half life	(Gross et al., 1988)
Intracellular trafficking	(Baenziger et al., 1988)
Glycoprotein activity	(Terasaki et al., 1988)
Aberrant blood group expression	(Yamada et al., 1988)
Malignant transformation	(Hakomori, 1985)
Glycoprotein immunoreactivity	(Pellegrini et al., 1988)

Pregnancy	(Avvakumev et al., 1988)
Antibody valency	(Margni et al., 1988)
Subunit assembly	(Matzuk et al., 1988)
Fetal development	(Wong et al., 1988)
IL 1 modulation	(Bories et al., 1987)
Membrane anchoring	(Fukuda et al., 1988)
Immunosuppression	(Prokazova et al., 1988)
Enzyme activation energy	(Delanghe et al., 1988)
Macrophage migration	(Gordon et al., 1987)

Tumor cells show aberrant cell membrane glycosylation (Warren et al., 1978; Yamada et al., 1978; Warren, 1981; Yogeeswaran, 1983; Fukuda, 1985; Debray et al., 1986; Bolscher et al., 1986; Gavazova et al., 1987; Itai et al., 1988). The neoexpression of certain gangliosides by fibroblasts after oncogene transfection points at a possible mechanism at the molecular level (Nakaishi et al., 1988). Tumors also produce plasma proteins (Yoshimura et al, 1978). As a result, the aberrant expression of blood group antigens and other carbohydrate determinants on the cell membrane (Tsuji, 1987; Hakomori, 1986) and in plasma of patients with cancer (Thompson et al., 1987) is observed, with diagnostic and therapeutic implications (Bernier et al., 1988). In this context it is of interest to note that a number of lectins have been found to posses anti-tumor activities, such as the lectins from mistletoe (Holtskog et al., 1988), from Loach eggs (Sarcophaga peregrina) (Yamazaki et al., 1984; Ohkuma et al., 1985) from wheat germ (Esumi-Kurisu et al., 1983), from Griffonia simplicifolia (Maddox et al., 1982; Eckhardt et al., 1982) and from bitter melon (Momordica charantia) (Jilka et al., 1983). Lectins may also have applications in other diseases (Levi et al., 1983).

A number of compounds have shown to have an influence on the glycosylation of glycoproteins and glycolipids, such as estrogens (Raynes, 1982; Dutt et al., 1988), monokines (Mackiewicz et al., 1987) retinoids and hormone-releasing hormones. All these agents may have a role as anticancer drugs. The influence on glycoprotein hormone glycosylation by glycoprotein-releasing hormones has recently been described (Taylor et al, 1986; Mori et al., 1986; Gesundheid et al., 1986, 1987). The use of releasing hormones (i.e. LHRH and analogs) in the therapy of prostatic cancer has been well established. Retinoids have a profound effect on the synthesis of carbohydrate chains (Lotan et al., 1987; Campbel et al., 1987; Reiss et al., 1985; Cope et al., 1984; Nakhasi et al., 1984; Kume et al., 1983; Deutsch et al., 1983; Ietten et al, 1983; Couch et al., 1988). They have been tried as experimental anti-tumor agents (Meromsky et al., 1984, Lotan et al., 1984) as well as anti-rheumatoid arthritis agents (Brinckerhoff et al., 1985; Bussière et al., 1988), diseases in which modified glycosylation patterns are involved (Hakomori, 1986, Pekelharing et al., 1988). Thus, the investigation of a number of glycosyltransferases in malignancy has

detected important changes in the relative activities of these enzymes (Yazawa et al., 1986). Carcinogens may induce certain enzymes involved in the synthesis of carbohydrate structures (Holmes et al., 1987).

The importance of carbohydrate structures is also indicated by the fact that the recently introduced tumor markers CA 125, CA 19-9, CA 50, CA 15.3, CA 1 (Oxford antigen), SSEA-1 and T-antigen are all of carbohydrate nature (Feizi, 1985; Hakomori, 1985; Hakomori et al., 1983, Smets et al., 1984; Itzkowitz et al., 1986). Simple tests for cancer-associated sugar moieties are being developed (Shamshuddin et al., 1988).

The investigation of cellular carbohydrate receptors (membrane lectins) during development and in tumor tissues is a new and exciting area, with promising results both for diagnosis and for therapy of cancer (Lotan et al., 1988; Raz et al., 1987; Gabius et al., 1988, 1987 a,b,c; Raz et al., 1981, 1984; Lotan et al., 1985; Gabius et al., 1985, 1985, 1985; Gabius et al., 1984; Roche et al., 1983; Aizawa et al., 1988). The inhibition of metastatic potential by certain saccharides and glycosidases underlines the importance of cell-cell recognition mechanisms by membrane lectin - carbohydrate interaction (Wright et al., 1988; Beuth et al., 1987).

Summarizing, the patterns of glycosylation of glycoproteins and glycolipids show interesting correlations with processes of development and disease, although their precise functions are largely unknown at present.

1.3.2. *Changes in plasma glycoprotein glycosylation*

Approximately ten years ago, using a rat hepatocyte plasma membrane inhibition assay, elevated levels of "serum desialylated glycoproteins" were detected in patients with hepatobiliary dysfunction (Marshall et al., 1978; Arima et al., 1976, 1977). In cirrhosis greatly increased quantities of a heterogeneous population of desialylated glycoproteins are present in serum, responsible for inhibition of the hepatocyte membrane assay (Marshall et al., 1978). The term "asialoglycoproteinemia" was introduced a few years later to describe a most unusual finding of a generalized absence of sialic acids from plasma glycoproteins in a patient with primary hepatic cancer (Sobue et al., 1980). Subsequently, a modified assay was developed to quantitate asialoglycoproteins in serum (Byrn et al., 1984). In patients with chronic liver disease or liver cell carcinoma "hyperasialoglycoproteinemia" was found (Sawamura et al., 1984). Hepatoma cells in culture indeed synthesize abnormally glycosylated glycoproteins (Carlson et al., 1984).

In the following paragraph the modifications are described of the glycosylation during development and disease of the few plasma glycoproteins for which this aspect has been recently investigated. These proteins include the tumor products α-fetoprotein (AFP) and chorionic gonadotropin (HCG) which are aberrantly glycosylated by the tumor cells, as well as ubiquitous plasma proteins such as IgG, transferrin and α_1-acid glycoprotein for which altered glycosylation has been described.

1.3.2.1. Transferrin

With respect to its glycan structure, transferrin is one of the best characterized glycoproteins. Human plasma transferrin contains two N-linked diantennary glycans, at aminoacid positions 413 and 611 (Spik et al., 1975; Macgillivray et al., 1982).

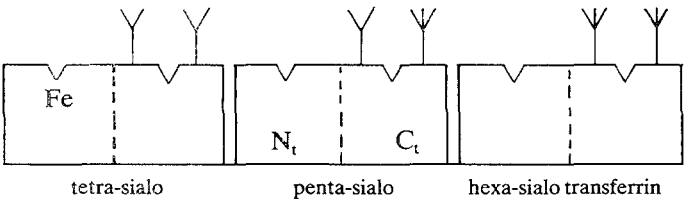


Figure 1-3. The glycosylation of human transferrin (from de Jong et al., 1987, with permission).

Small amounts of tri-antennary and even tetra-antennary chains do occur (Wong et al., 1978; Marz et al., 1982; Spik et al., 1985). Thus, 4-sialo transferrin is the major compound, followed by the 5-sialo and 3-sialo variants. Other sialo variants occur as minor fractions in plasma as well as in cerebrospinal fluid, amniotic fluid and synovial fluid, although the various patterns (obtained with crossed immunoelectrofocusing) are clearly different (van Eijk et al., 1983; Arrer et al., 1987). In contrast to cell membrane glycans, the half-life of the plasma transferrin glycan probably equals that of the protein part of molecule (Josic et al., 1987).

Changes in the glycosylation of plasma transferrin have been described (Jaeken et al., 1984; van Eijk et al., 1987; Bierings et al., 1987; Poupon et al., 1985; Stibler et al., 1986; van Noort et al., 1987). As the liver is the major transferrin producing organ (but not the only one (Kitada et al., 1985; Lum et al., 1986; Yoshimura et al., 1978; Morrore et al., 1988)), liver pathology may lead to a different distribution of the transferrin subfractions. Changes in tissue carbohydrate receptors, leading to changes in half-lives of glycoprotein subfractions, may also play a role as well as alterations in biosynthesis.

Hepatoma cell cultures secrete abnormally glycosylated proteins such as transferrin, α_1-antitrypsin, α_1-chymotrypsin and α_1-acid glycoprotein (Carlson et al., 1984; Alm et al. 1985). In patients with hepatoma this defect is not easy to recognize because the proportion of hepatoma-produced glycoproteins is small compared to their normally glycosylated counterparts produced by the healthy part of the liver. One case has been described of a hepatoma patient producing very large amounts of asialoglycoproteins, enough to be detected with immunoelectrophoresis (Sobue et al., 1980), a technique which is usually too insensitive in detecting these modifications.

In patients with cirrhosis, a disease affecting the liver as a whole, the less sialylated transferrin subfractions with pI values of 5.7 and 5.9 are significantly

elevated (Poupon et al., 1985). A method has been described to quantitate these fractions in the clinical setting (Stibler et al., 1986). On the other hand, liver cancer plasma samples were not different from controls. This finding does not exclude abnormal glycosylation, as higher branching but incomplete sialylation may lead to a normal distribution pattern with respect to isoelectric points. Uncharged additional sugar groups are not detected using methods based on charge differences. They may be detected by binding studies with lectins that are sensitive for the branching pattern of the glycans.

Stibler and Borg showed the incomplete sialylation of transferrin from alcoholics in a number of studies (Stibler et al., 1981, 1986). Others have confirmed these findings using chromatofocussing, indicating an increase in the pI 5.7 component (Storey et al., 1987; Petren et al., 1988). Carbohydrate analysis of purified whole transferrin showed that not only were sialic acids missing, but also some galactose and N-acetyl-glucosamine units. Even the mannose content was diminished (Stibler et al., 1986). The different reductions of these sugars indicated that apart from incomplete synthesis, an altered structural composition may also occur. Using chromatofocusing, desialylated transferrin in serum was separated from normal transferrin. Only in alcoholic subjects were values raised above 2% as measured by radioimmunoassay (Storey et al., 1987). Notably, no raised values were found in non-alcoholic steatohepatitis patients, in diabetes patients and in non-alcoholic liver disease patients. The results from patients with steatohepatitis were of particular interest because their liver disease is morphologically indistinguishable from alcoholic hepatitis. The test for desialylated transferrin compared favourably with routinely used blood tests such as MCV, γ-GT, uric acid, ASAT, ALAT, LDH, alkaline phosphatase and HDL (Storey et al., 1987; Gjerde et al., 1988). It is the first test to provide a retrospective quantitation of alcohol consumption, reminiscent to the Hba1c assay as a long-term control for glucosemia in diabetic patients. Using isoelectric focusing and crossed immunoelectrophoresis in the second dimension a shift to higher sialylated forms of transferrin was noted in various other diseases (van Eijk et al., 1987). Other charged molecules or abnormal linkages may also be involved (Narita et al., 1987).

1.3.2.2. Alpha-Fetoprotein

AFP is a glycoprotein of 69000 Dalton molecular weight. It contains one N-linked diantennary glycan which is responsible for the heterogenity of AFP. Fetal production starts in the yolk sac, where the Con A non-binding form is produced having a bisected diantennary glycan. In the second trimester of pregnancy the fetal liver takes over and produces AFP of the Con A binding form having a non-intersected biantennary glycan. This is an example of tissue-specific glycosylation. After 33 weeks AFP synthesis decreases sharply with a concomitant increase in the synthesis of a very similar but non-glycosylated protein: albumin.

Apart from a change in Con A binding due to a shift from a intersected to a non-intersected glycan, the Lens culinaris (LCA) binding changes as well (Toftagar-Larsen et al., 1983). As LCA binds fucosylated diantennary glycans (Kornfeld et al., 1981) the observed reduction in binding indicates a shift in α1-6 fucosylation of the first N-acetyl-glucosamine of the glycan towards less fucose incorporation.

Abnormalities in the developmental changes of both the Con A and the LCA binding AFP fractions have been found in a number of neural tube defects and other fetal abnormalities (Toftagar-Larsen et al., 1983; Buamah et al., 1981; Kolho et al., 1983; Mackiewicz et al., 1984). Thus, the quantitation of the Con A and LCA binding fractions has been devised as an ancillary diagnostic test in pregnancy, next to the AFP concentration in serum or amniotic fluid, although Con A-results have been challenged (Jones et al., 1988).

In adults, AFP reappears in serum of patients with primary hepatomas and germ cell tumors, especially endodermal sinus tumors. This observation has led to the idea of oncofetal gene expression. However, the relationship with histological findings is inconsistent. AFP-producing and non-producing cells may be present in the same tumor. Some tumors produce AFP but do not secrete the material into the circulation. (This may be caused by aberrant AFP glycosylation). Teratocarcinomas often consist of a mixture of cell types, some of which have been shown to produce AFP while others do not. Hepatoblastomas on the one hand, and pure endodermal sinus tumors on the other always seem to produce AFP. The glycosylation of AFP in the two groups is different. Yolk sac tumor AFP is Con A non-binding because it contains an intersecting GlcNac, just as fetal yolk sac AFP (Yamashita et al., 1983; Tsuchida et al., 1984). Hepatoma-produced AFP is glycosylated the same as fetal liver AFP: it contains a fucosylated diantennary glycan (Yoshima et al., 1980).

Using three galactose specific lectins (from Allomyrina dichotoma, from Ricinus communis and from Phaseolus vulgaris) large differences in reactivity of AFP fractions were found. Tumor associated fractions were detected but patterns varied between patients indicating heterogeneity between tumors (Taketa et al., 1986). In conclusion, the differential reactivity of serum AFP towards Con A and lentil lectin provides a simple test which has its place in the diagnosis of various liver diseases (Buamah et al., 1987; Aoyagi et al., 1988; Breborowicz, 1988).

1.3.2.3. α_1-acid glycoprotein (AGP)

AGP is an unusual plasma glycoprotein. It has an extremely low pI of 2.9, partly due to the large number of sialic acids present on the molecule. AGP contains 5 highly branched N-linked glycans; its carbohydrate accounts for 45% of its molecular weight of 44.000 daltons (Fournet et al., 1978). It is an acute phase protein with normal plasma concentrations between 0.6 and 1.4 g /l.

In a number of diseases the glycan composition of AGP is modified (Moule

et al., 1987; Bleasby et al., 1985). In hepatic disorders desialylation of AGP is found (Serbource-Goguel et al., 1983). A number of cancers induce altered lectin binding as shown with crossed affinoimmunoelectrophoresis (Hanson et al., 1984) indicating changes in the di-triantennary glycan ratio. The glycan composition of AGP isolated from liver metastases of lung, colon and breast tumors is different from plasma AGP. α1-6 fucose linked to the diantennary chain is also a unique finding (Chandrasekaran et al., 1984). In patients with rheumatoid arthritis significant changes in the microheterogeneity patterns were observed (Mackiewicz et al., 1987).

The physiological role of AGP is unclear. It has chemokinetic and immunosuppressive properties, dependent on the composition of the carbohydrate groups (Castello et al., 1984; Cheresh et al., 1984; Bennett et al., 1980). In the pig, AGP is the major fetal serum protein (50% of total), decreasing to 0,3% in the adult pig (Lampreave et al., 1984). This development-dependent change in concentration is accompanied by an increase in fucose content and in Con A binding. The modifications of the N-glycans of four acute phase proteins in patients with severe burns was recently investigated (Mallet et al., 1987). A major shift towards diantennary glycans in α_1-acid glycoprotein as well as in α_1-antitrypsin was detected using Con A-Sepharose column chromatography.

1.3.2.4. Immunoglobulin G (IgG)

The plasma of patients with rheumatoid arthritis (RA) contains aggregated immunoglobulins (Mannik et al., 1985). These so-called "immune-complexes" differ from antigen-antibody complexes in several ways. Because of the absence of antigen it is suggested that the aggregates consist of certain immunoglobulins with affinity for other immunoglobulins (autoantibodies). The strength of the interaction between the immunoglobulins ($Ka = 10^5\ M^{-1}$) is less than that observed for normal antigen-antibody interactions, indicating that a different type of interaction may be involved.

Immunoglobulin G is a major plasma glycoprotein containing, on average, 2.8 N-linked carbohydrate chains per molecule of which 2 are located in the Fc fragment (Rademacher et al., 1984; Mizuochi et al., 1982). The carbohydrate chains consist of a variety of diantennary complex-type structures, some containing an extra "bisecting" N-acetylglucosamine or a fucose residue (Mizuochi et al., 1982).

The recent finding of a reduced galactosylation of the diantennary carbohydrate chains of total IgG (Parekh et al., 1985; Pekelharing et al, 1988; Tomana et al., 1988) in patients with RA, due to a reduced B-cell galactosyltransferase (Axford et al, 1987) has led to a new hypothesis concerning the aggregation of IgG molecules (Parekh et al., 1985). Less sugar constituents in the N-linked glycans of IgG could make IgG more "sticky" due to a lectin-like activity.

IgG from RA patients bound significantly more to the immobilised plant lectins Concanavalin A and Peanut agglutinin than IgG from normals (Malaise

et al., 1986, 1987). This could either be due to a modified IgG glycosylation (Parekh et al., 1985), or due to an altered conformation of IgG (Hansson et al., 1985) or both, leading to a change in lectin binding. Our own results (chapter 5.2) suggest a decrease in IgG galactose content in RA patients and an increase during pregnancy, which might explain the temporary remission of RA commonly observed during gestation (Pekelharing et al., 1988)

1.3.2.5. Human Chorionic Gonadotropin (HCG)

HCG is a glycoprotein hormone produced by the placental trophoblast and by choriocarcinoma tissue, and secreted into serum and urine. It is composed of two dissimilar subunits, α and β, joined non-covalently. The common α subunit also occurs in the hormones LH, FSH and TSH, while the hormone-specific β units are different (Green et al., 1986). Both α- and β-subunits contain two asparagine-linked sugar chains, the β-subunit of HCG contains an additional four O-linked sugar chains (Pierce et al., 1981). The carbohydrate residues appear to be essential for the biological activity of the hormones. (Sairam et al., 1985; Manjunath et al., 1982; Rosa et al., 1984; Kalyan et al., 1983; Baenziger et al., 1988).

The asparagine-linked sugar chains of choriocarcinoma HCG are quite different from the normal hormone (Mizuochi et al., 1983; Imamura et al., 1987; Takeuchi et al., 1984; Koyama et al., 1986; Cox, 1986; Nishimura et al., 1985; Yazaki et al., 1987; Endo et al., 1987, 1988). The number of sialic acid groups is highly decreased or increased, while unique triantennary glycans are present together with anomalous biantennary chains. Increased α1-6 linked fucose was also found. By contrast, the HCG purified from patients with hydatiform mole, one of the trophoblastic diseases, gave almost the same profile of asparagine-linked sugar chains as that of normal HCG.

A method designated by the authors as a lectin-immunoradiometric assay (LIRMA) was developed to quantitate desialylated forms of HCG in urine (Imamura et al., 1987) Both with RCA, which recognises Galβ1-4GlcNAc, as with PNA, which recognises Galβ1-3GalNAc, elevated levels were found in patients with choriocarcinoma compared to pregnant women and hydatiform mole patients. This indicates loss of sialic acids both from the N-linked as well as the O-linked glycans (Endo et al., 1988).

The major difference in O-glycosylation of HCG or its subunits produced in choriocarcinoma and in pregnancy appears to be the abundance of the hexasaccharide NeuAcα2-3Galβ1-3(NeuAcα2-3Galβ1-4GlcNAcβ1-6) GalNAc in tumor HCG (Cole, 1987 a,b).

A major question left to be answered concerns the link between malignancy and abnormal glycosylation of cell membranes and secretory proteins (Kobata, 1988). The glycosylation of most proteins produced by tumors studied thus far suggest that this relation indeed exists. But is it a prerequisite or an epiphenomenon?

1.3.2.6. Fibrinogen

Fetal fibrinogen contains more sialic acids than the adult form, as a function of gestational age (Francis et al., 1982; Galanakis et al., 1983). It also contains more phosphate while being less active in the clotting process (Witt et al., 1982). At birth the major difference between fetal (cord) and adult fibrinogen appears to be the 3-4 times higher phosphorus content of cord fibrinogen (Hamulyak et al., 1983). The ESR changes in pregnancy may partly be due to an abnormal fibrinogen glycosylation (Ozanne et al., 1983). Fibrin formation by thrombin is accelerated when less sialic acids are present on fibrinogen (Galanakis et al., 1983; Martinez et al., 1983a; Diaz-Maurino et al., 1982). Others report no differences in clotting activity between the glycosylated and non-glycosylated forms of fibrinogen (Gilman et al., 1984).

In liver disease fibrinogen contains more sialylated (Martinez et al., 1983a) and more branched glycans (Martinez et al., 1983b). Especially during acquired dysfibrinogenemia of liver disease the sialic acid content of fibrinogen is increased considerably (Martinez et al., 1983). In liver cirrhosis the modified fibrinogen with its higher sialylation shows characteristics similar to those of fetal fibrinogen (Narvaiza et al., 1986). Increased sialyltransferase might be at the basis of the increased sialic acid content (Francis et al., 1986; Simmonds et al., 1987).

From this short overview it is concluded that techniques to quantify the different glycosylation states of plasma glycoproteins would be helpful in a number of instances, both in diagnosis and for monitoring therapy. It is a logical choice to develop techniques using lectins: the carbohydrate binding proteins of non-immune origin.

1.4 Use of lectins in sandwich ELISA techniques

In clinical chemistry, a very sensitive and specific assay is the enzyme-linked immunosorbent assay (ELISA), first described by van Weemen et al. (1971) and Engvall et al. (1972).

Basically, it consists of an antibody coated solid phase provided by plastic tubes, balls or microtiter plates. After incubation with a nonsaturating amount of the protein to be measured, a second antibody linked to a marker enzyme is added. A standard curve relates the amount of protein present in the sample to the amount of enzyme activity bound to the tube. This type of assay is called the "sandwich" or two-site ELISA.

In theory, by replacing either the immobilized antibody or the enzyme-linked antibody with a lectin or other carbohydrate-binding protein, it is possible to create a heterologous Lectin-Enzyme Immuno-Assay system (LEIA) to study the glycosylation of a glycoprotein (fig. 1-4). The "sandwich" now consists of a lectin and an antibody. Only the protein that meets two conditions is measured;

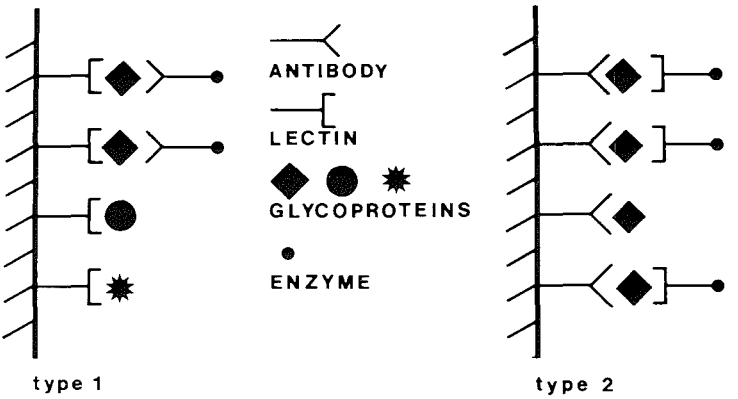


Figure 1-4. The two types of "sandwich" Lectin Enzyme Immuno-Assay (LEIA) using immobilized lectin (type 1) or enzyme-linked lectin (type 2).

i.e. it must be the right glycoprotein to be bound by the antibody, and it must have the right glycan structure to bind the lectin used. The lectin may replace either the immobilized or the enzyme-bound antibody.

In the first type of assay a lectin is immobilized and binds the glycan chain of the glycoprotein in question. The amount of protein is subsequently quantitated using the enzyme-linked antibody as in the ELISA-technique. This type of assay has a potential drawback. The immobilized lectin also binds glycans which are connected to other glycoproteins, thus creating a "cross-reactivity" with other glycoproteins present in the sample. This could strongly influence the results for the glycoprotein under study.

In the second type of assay the antibody-enzyme conjugate is replaced by a lectin-enzyme conjugate. Antibodies to the glycoprotein are immobilized on the solid phase as in the ELISA technique. In contrast to the ELISA, saturating amounts of the glycoprotein should now be used, after which the lectin reactive fraction is quantitated by adding the lectin-enzyme conjugate. The drawback of the first type of assay, interference from other glycoproteins in the sample, may not be present here, because in theory the immobilized antibodies can only bind the glycoprotein against which they were raised. The sensitivity of the assay should be better as more glycoprotein material is captured in this system. Thus, the second type has an important advantage over the first, because in theory, purification of the glycoprotein is not necessary. During the incubation of the sample with the immobilized antibody only the specific glycoprotein antigen is recognized and bound, and all other proteins remain in solution and are washed away. In principle, therefore, this assay should also be able to investigate the glycosylation of an individual glycoprotein present in a complex mixture such as human plasma. However, as the non-specific binding of enzyme-linked lectin to coated antibody immunoglobulins was considered to be a substantial drawback to using the type 2 system, the type 1 system was first investigated.

1.5 Aim of the study

The aim of this work is to determine if lectins can be used in "sandwich" ELISA techniques so that the glycosylation of specific proteins in mixtures could be characterised in a fast and sensitive way without prior purification of the protein. Furthermore, the feasability of lectin-enzyme binding assays will be explored in various ways to assess the lectin specificity, and to detect the binding of lectins to normal plasma glycoproteins and their disease-associated glycoforms.

CHAPTER 2

PROSPECTS FOR LECTIN-ENZYME IMMUNOASSAYS USING A LECTIN AS THE IMMOBILISED BINDER, INVESTIGATED BY LECTIN COLUMN CHROMATOGRAPHY OF NORMAL HUMAN PLASMA

2.1. Introduction

For the development of enzyme-immunoassays with a lectin as the immobilised binder, two criteria must be met. Firstly, under normal conditions, very few glycoproteins in plasma must have carbohydrate structures to which the utilised lectin can bind. A review of the literature suggested that sialic acid loss from glycoproteins with concomittant terminal galactose expression occured in certain liver diseases. Thus, it was reasoned that with galactose-binding lectins as immobilised binders in a two-site lectin-immunoassay it would be possible to quantitate sialic acid loss from plasma glycoproteins. Ideally the lectin used should bind only to the incompletely sialylated plasma glycoprotein in question, without binding to other asialo glycoproteins and without binding the normal fully sialylated glycoproteins. An enzyme-linked antibody could then simply detect and quantitate the amount of (galactose-terminal) glycoprotein bound, as in the ELISA-system. As terminal galactose expression in normal plasma seemed to be quite low (Byrn et al., 1984) the problem of "cross-reactivity" and inhibition by other galacto-glycoproteins present in plasma was expected to be low.

Secondly, the lectin used must adhere onto plastic tubes or microtiter plates and still show its binding characteristics. This was not considered to be a problem as many glycoproteins (such as immunoglobulins) adhere to plastic without losing their binding affinity or other characterstics. Lectins show quite a peculiar reaction pattern with glycoproteins. The precipitation of ten galactose-specific lectins with purified plasma asialo glycoproteins in gel diffusion experiments was determined by Uhlenbruck et al. (1978) (table 2-1).

No clear pattern with respect to binding preference can be seen. However, the number of glycans linked to the glycoproteins seems to play a role. If the results are presented with respect to the estimated number of glycans per protein (table 2-2) it becomes clear that this characteristic is important in lectin-glycoprotein interactions.

It appears that the more glycans a protein has, the more chance it has to overcome spatial restrictions in the binding to lectins that otherwise inhibits precipitation in gels. Alternatively, due to the weak interaction of lectin and glycan (with a typical Ka of 10^4 M^{-1}) it may be a prerequisite for some lectins

Table 2-1. Precipitation of asialo glycoproteins by lectins (Uhlenbruck et al., 1978; Luther et al., 1980).

Serum glycoprotein after neuramindase-treatment	Precipitating lectins from Tridacna maxima	Tridacna gigas	Rutilus rutilus	Ricinus communis
Antithrombin III	+	-	-	+
Haemopexin	++	+	-	+
Transferrin	-	-	-	+
α_1S-Glycoprotein	+	-	-	++
Fetuin	+	-	-	++
β_2-Glycoprotein	++	+	-	++
Cholinesterase	++	+	++	++
α_1B-Glycoprotein	-	-	+	+
3,1S-α_2-Glycoprotein	++	-	-	++
α_1-Antitrypsin	-	+	-	++
α_2HS-Glycoprotein	-	-	-	+
α_2-Macroglobulin	++	+	+	++
α_1-Antichymotrypsin	++	+	++	++
8S-α_3-Glycoprotein	++	++	++	+
β_2-Glycoprotein III	-	-	++	-
Gc-Globulin	-	-	-	+
Secretory component	++	+	+	++
Lactoferrin	++	+	-	+
Prothrombin	-	-	++	+
Inter-α-trypsininhibitor	+	-	++	++
C3-Activator	++	+	-	++
3,8S-α_2-Glycoprotein	-	-	-	++
Coeruloplasmin	-	+	++	++
C1-Inactivator	+	+	++	++
9,5S-α_1-Glycoprotein	-	-	++	++
Haptoglobin	++	+	+	++
Thyroxin binding globulin	++	+	+	++
IgA	+	-	-	++
IgM	+	+	+	+
IgD	-	-	+	++
IgG	+	-	-	-
IgE	++	++	+	++

- = no pricipitin reaction

+ = weak, but good visible precipitin reaction

++ = strong, sharp precipitation line

* = precipitation identical with both native and neuraminidase-treated glycoproteins

nd = not done

Glycine soja	Ononis spinosa	Axinella polypoides	Abrus precatorius	Wistaria floribunda	Viscum album
-	-	-	+	+	+
++	++	-	+	+	-
-	-	-	-	nd	+
+	++	-	+	-	+
+	++	-	+	+	+
++	++	++	++	+	-
++	++	+	++	+	+
-	-	-	+	nd	nd
+	++	-	++	-	-
-	-	-	+	-	-
-	-	-	+	+	-
+	++	++	++	+	+
++	++	-	++	+	+
++	++	-	++	+	+
-	-	-	-	-	-
-	-	-	-	-	-
++	++	-	++	nd	nd
+	++	-	++	-	-
-	+	-	-	-	+
+	++	-	+	+	+
+	-	+	++	-	-
-	-	-	+	+	-
+	+	-	+	+	+
+	++	-	++	+	+
-	-	+	++	-	+
++	++	-	++	+	+
+	++	-	++	+	-
-	++	-	++	+	+
-	++	-	++	+	+
-	-	-	+	-	+
-	-	-	-	-	+
++	++	++	++	+	+

LECTIN		GC		TF		α2	β2	AAT		AT		3,8S		α1	3,1S		I	Cer		IgD		IgE		CA
	β2 III		α1 B		IgG	HS	I		TBG	III	C3 act	α2	Hpx	S	α2	IgA	T		Hap		ACT		9,5S α1	inact
	1	1	2	2	2	3	3	3	3	4	4	4	5	5	5	5	6	6	6	6	7	10	11	18
AXINELLA POLYPOIDES							++				+											++	+	
TRIDACNA GIGAS							+	+	+		+	+						+	+		+	++		+
RUTILUS RUTILUS	++		+						+								++	++	+	+	++	+	++	++
GLYCINE SOJA							++		+		+	++		+	+		+	+	++		++	++		+
WISTARIA FLORIBUNDA			.	.			+		+	+		+	+			+	+	+	+		+	+		+
VISCUM ALBUM			.	+	+	+				+				+		+	+	+	+	+	+	+	+	+
ONONIS SPINOSA							++		++				++	++	++	++	++	+	++		++	++		++
TRIDACNA MAXIMA					+		++		++	+	++		++	+	++	+	+		++		++	++		+
ABRUS PRECATORIUS			+			+	++	+	++	+	++	+	+	+	++	++	+	+	++	+	++	++	++	++
RICINUS COMMUNIS		+	+	+		+	++	++	++	+	++	++	+	++	++	++	++	++	++	++	++	++	++	++

estimated number of glycans per molecule

Table 2-2. Asialo glycoprotein precipitation by lectins in relation to the number of glycans of the glycoproteins (adapted from Uhlenbruck et al., 1978). (· = not done).

to have multiple interactions before precipitation occurs. However, the sensitivity for the number of glycans differs between lectins.

To study the terminal galactose expression of plasma glycoproteins and to find a lectin with the desired specificity the binding of normal plasma glycoproteins and their asialo derivatives to a number of galactose specific lectins was determined. We used nine galactose-specific lectins immobilised to Sepharose to fractionate plasma glycoproteins according to their galactose expression. Both the bound and the unbound fraction were analysed with crossed immuno-electrophoresis to detect and classify any plasma glycoprotein with affinity for the immobilised lectin. A laser nephelometer was used to quantify the concentration of the most common proteins in both fractions.

2.2. **Materials and Methods**

The following lectins were used, either immobilised (5 mg/ml gel) by the supplier (E.Y. Laboratories, San Mateo, Calif., USA) or in our laboratory (*) using CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden):

* Tridacna squamosa (TSA)
* Crotalaria juncea (CJA)
* Bandeira simplicifolia I (BSI)
  Ricinus communis (RCA) Maclura pomifera (MPA)
  Abrus precatorius (APA)
  Sophora japonica (SJA)
  Arachis hypogaea (PNA)
* Vicia villosa (VVA)

For immobilisation of lectins the following procedure was used: 0.6 gr CNBr-Sepharose was washed for 15 minutes in 1 mM HCl, then washed with buffer A (0.1 M $NaHCO_3/Na_2CO_3$ + 0.5 M NaCl, pH 8.3). Lectin was added (10 mg in 4 ml buffer A) and incubated for 2 hours at room temperature and for 16 hours at 4°C. Remaining active groups were blocked with 5 ml 0.1M ethanolamine (pH 8.0). The gel was then washed 4x, alternating with buffer A and buffer B (0.1 M acetate buffer, pH 4.0). After a final washing with PBS (0.1 M phosphate buffer, pH 6.8 with 150 mM NaCl) the gel (2 to 3 ml) was allowed to settle in a glass column (internal diameter 7 mm). As a reference a column with unconjugated Sepharose was used. Lectin column chromatography: 0.3 ml of a normal human serum pool, either untreated or neuraminidase treated, was brought onto the column and incubated for 30 minutes at room temperature. Before and after application of serum the column was washed with 0.1 M Tris buffer, pH 8.0, containing 1 mM Ca^{++} and 0.1 mM Mn^{++}. Fractions were monitored at 254 nm (Uvicord, LKB Produkter, Bromma, Sweden). Bound material was eluted with 5 ml Tris buffer containing 100 mM galactose, after which the column was regenerated and washed with 0.1 M acetate buffer, pH 4.5.

Crossed immunoelectrophoresis was run according to standard procedures. Briefly, a 1 mm thick 1% agarose gel was used in 37.5 mM veronal buffer, pH 8.6 on glass plates (9.4 x 8.2 cm). Sample volume was 5 μl of the unbound or bound fractions. A Multiphor electrophoresis unit (LKB) was used: the first dimension was run for 1 hour at 300 Volts, the second dimension for 5 hours at 100 Volts (after application on the gel of polyvalent antiserum diluted 1:5 or 1:10). Patterns were interpreted by visual inspection.

Kinetic immunonephelometry was performed using a Baker 420 laser nephelometer (Baker Instruments, Allentown, USA) according to the manufacturers instructions. The antisera were obtained from Behringwerke, Marburg, Germany. We analysed the unbound and bound fractions and the untreated normal serum for their concentrations of transferrin (TF), α_1-acid glycoprotein (AGP), haptoglobin (Hp), IgA, IgG and albumin (Alb). Albumin served as an internal reference to detect any carry-over during lectin column chromatography from the unbound to the bound fraction. As it contains no carbohydrate, it should be found in the unbound fraction only. Immobilised neuraminidase (1 U) from Vibrio cholerae (Behringwerke) was used to prepare asialo-serum glycoproteins: 0.3 ml normal serum was incubated on the column in 0.05 M sodium acetate buffer pH 5.5 containing 0.15 M NaCl and 9 mM Ca^{++} for 1 hour at room temperature.

2.3. Results

Serum glycoproteins did not bind to Sepharose or to Sepharose conjugated with any of the following lectins: BSI, SJA, VVA, PNA and TSA. Elution of these columns with galactose produced no absorption peak at 254 nm and no precipitation arc was found in the crossed immunoelectrophoretic analysis of the "bound" fraction, and no arc was found to be absent using this analysis in the unbound fraction. By contrast, some proteins are bound to the other lectins (table 2-3).

Table 2-3: Analysis of sialo- and asialo glycoproteins from normal serum separated on immobilised galactose-binding lectins

Lectin	Crossed immunoelectrophoresis		Laser nephelometry					
	Present in bound fraction	Missing in unbound fraction	Percent of protein present in bound fraction (in parentheses: neuraminidase treated serum)					
			TF	AGP	Hp	IgA	IgG	Alb
RCA	weak α_1 band three α_2^1 bands two γ^2 bands	α_2 bands	0 (100)	0 (100)	100 (100)	70 (100)	10 (10)	0 (0)
MPA	γ band	γ band	0 (0)	0 (0)	0 (0)	70 (100)	0 (0)	0 (0)
APA	two weak α_2-bands	-	0 (trace)	0 (0)	trace (trace)	0 (0)	0 (0)	0 (0)
CJA	weak band in Albumin region	-	0 (0)	0 (0)	trace (100)	0 (0)	0 (0)	0 (0)

Although all lectins used are galactose specific (that is: are best inhibited by galactose), they displayed large differences in their affinity for (asialo) glycoproteins. This observation has been reported earlier for lectin-glycoprotein precipitation during "affino-diffusion" in gels using purified neuraminidase-treated serum glycoproteins (Uhlenbruck et al., 1978).

Of the lectins tested, Ricinus communis agglutinin binds to the most plasma glycoproteins. It binds all haptoglobin and a major portion of IgA from normal serum. This could be IgA_1, since the IgA binding galactose-specific lectin ("Jacalin") from jackfruit (Artocarpus integrifolia) (Roque-Barreira et al., 1985) seems to bind only to IgA_1 (Kondoh et al., 1986). Besides binding to some IgG, possibly IgG_3 (Saltvedt et al., 1975), immunoelectrophoresis showed binding to at least two other as yet undefined glycoproteins present in normal serum, probably α2-macroglobulin, ceruloplasmin or IgM (Surolia et al., 1975; Harboe et al., 1975). Using neuraminidase-treated serum the unselective binding of RCA to asialo glycoproteins was confirmed: it binds to all of the TF, AGP, Hp and IgA present.

The other lectins are more selective than RCA. MPA binds only IgA. APA binds only traces of transferrin and haptoglobin, and CJA binds a trace of haptoglobin and binds asialohaptoglobin.

2.4. Discussion

The original idea of a galactose-specific lectin as the immobilised binder in a lectin enzyme immunoassay for galacto- transferrin was based on the assumption that the lectin does not bind normal plasma glycoproteins, whilst still binding the glycoprotein under study, which has a loss of sialic acids. However, depending on which galactose-specific lectin is chosen for the experiments, both substantial binding to other sialoproteins than transferrin and inability to bind to asialo-transferrin can be observed.

This means that non-saturating conditions should be used to avoid competition in the lectin enzyme immunoassays for the limited number of immobilised lectin molecules by other galacto-glycoproteins in plasma. Since we found such extensive plasma glycoprotein galactose expression, as measured with a number of galactose lectins, this indicated that large dilutions of sample were necessary. Given the sensitivity of ELISA techniques, this meant that only massive sialic acid loss from transferrin could be detected in a system which makes use of immobilised galactose-specific lectins. As shown in table 2-3, there is one combination of lectin and (asialo) glycoprotein in which type 1 lectin-enzyme immunoassay might work. Crotalaria juncea lectin binds only one (undetermined) normal plasma glycoprotein. Of the six major plasma glycoproteins in their asialo form only haptoglobin binds.

The agarose-linked lectins RCA and PNA were used with ^{125}I-labeled anti-HCG in a lectin-immunoradiometric assay (LIRMA) to quantitate urinary desialylated HCG in patients with choriocarcinoma (Imamura et al., 1987).

Significantly elevated levels were found in comparison with normal pregnancy or hydatiform mole. The binding of the radiolabeled antibody to the immobilised lectin was approx. 10% of the maximum signal (highest standard) in the case of PNA, and 20% in the case of RCA, just low enough to allow this method to be used. Interference from other glycoproteins in urine with terminal galactose groups was not investigated. The same approach was taken to detect changes in IgG glycosylation. The lectins Con A and PNA were coated onto microtiter plates and the amount of bound IgG quantitated with ^{125}I-Protein A or ^{125}I-anti-IgG (Malaise et al., 1986, 1987). However, the IgG was exhaustively purified from plasma before those binding studies.

2.5 Conclusions

Application of a lectin as the immobilised binder in lectin enzyme-immuno-assays is only possible when the lectin does not bind to any of the normal plasma glycoproteins, but binds (only) to the aberrantly glycosylated form of the glycoprotein under study. It is uncertain whether such a lectin will ever be found for the quantification of either galacto-transferrin, or any other abnormal glycosylated plasma glycoprotein. The method may be applicable, however, to the study of the glycosylation of purified glycoproteins.

Subsequently, the second type of lectin-enzyme immunoassays is explored, consisting of antibody-coated tubes or microtiter plates and using lectin-enzyme conjugates as the soluble "second" binder.

CHAPTER 3

THE DEVELOPMENT OF A LECTIN-ENZYME IMMUNOASSAY FOR TRANSFERRIN GLYCOFORMS WITH TERMINAL GALACTOSE GROUPS USING IMMOBILISED ANTI-TRANSFERRIN AND ENZYME-LABELED GALACTOSE-BINDING LECTIN FROM RICINUS COMMUNIS

3.1. **Introduction**

In this chapter a number of experiments are described in chronological order leading to the final protocol for lectin-enzyme immunoassays, using immobilised antibodies and lectin-enzyme complex. In the following paragraphs the reasons are given for the choice of transferrin, Ricinus communis lectin and the streptavidin-biotin system in the development of the principles of lectin-enzyme immunoassays.

3.1.1. *The choice of transferrin as the model compound*

The following considerations have led to the choice of transferrin as the model compound. First, it is a relatively abundant plasma glycoprotein (1.5 - 3.5 g /l), and it is easy to isolate milligram quantities. Second, the structures of its two N-linked glycan chains are well described. Most transferrin molecules have two diantennary side chains terminating in sialic acid residues: 4-sialo transferrin is by far the major fraction in humans, followed by the 3-sialo and 5-sialo fractions. Also, the glycans of transferrin are relatively simple; neither "intersecting" glucosamines nor α 1-2 or α 1-6 fucose residues that may influence lectin binding have been described to date. Third, the transferrin sialovariants are easily prepared and separated from each other by anion exchange chromatography. In this respect transferrin differs from a number of other plasma glycoproteins, in which sialic acid groups contribute less to the pI. The change in pI of transferrin due to the addition or removal of one sialic acid group is approx. 0.15 pH unit, large enough to allow a separation using chromatofocusing (Pekelharing et al., 1987a) This contrasts to other proteins where the difference between the sialovariants is much less than 0.1 pH unit. During this work, fast separation methods of the transferrin sialovariants were developed using the Pharmacia Fast Liquid Chromatography (FPLC) Mono P chromatofocusing and Mono Q anion-exchange columns (chapter 3.2).

On the other hand, transferrin as a model compound also has a disadvantage.

Not all transferrin molecules are loaded with iron. The iron ions (one or two) also contribute considerably to the isoelectric point. This problem was overcome by consequently saturating transferrin in all samples with iron, thus eliminating charge differences due to differences in iron content.

3.1.2. *The choice of Ricinus communis agglutinin as the lectin*

In choosing a lectin to develop the principles of the lectin-enzyme immunoassay, we limited ourselves to a number of commercially available galactose binding lectins. Many others have been described, originating however from rare organisms or seeds that are not available in this country.

We had three reasons to focus on the lectin from the castor bean (Ricinus communis). The RCA I (or RCA 120) lectin has a broad binding specificity; precipitating with 30 out of 32 asialoglycoproteins prepared from human plasma (Uhlenbruck et al., 1978). Nine other galactose specific lectins tested reacted with 6 to 27 proteins. RCA I binds to terminal galactose groups. It does bind to asialotransferrin. Only three other lectins are known to bind to (fractions of) asialotransferrin: the lectins of Viscum album (VAA), Vigna unguiculata (VUA) and peanut (PNA) (Uhlenbruck et al., 1978). Moreover, the binding constants of RCA I lectin and different glycopeptides has been relatively well established (Baenziger et al., 1979). It is unknown to what extent these observations also apply to protein-bound glycans.

A definitive disadvantage of RCA is its extreme toxicity when the material also contains the RCA toxin called ricin. This is a galactose specific lectin of 60,000 daltons molecular weight (also called RCA II or RCA 60 lectin), while the RCA I galactose lectin present in the Ricinus communis seeds has a molecular weight of 120,000 daltons. The toxin is probably the most toxic substance known: one molecule may completely block protein synthesis in a cell, leading to cell death. A few micrograms are enough to kill a man. This characteristic has been used in London in 1978 in the homicide of a Bulgarian dissident (Knight, 1979). In practice, the toxicity does not have to be a problem as only very small amounts of extremely diluted solutions are used in lectin-enzyme immunoassays.

3.1.3. *The choice of the streptavidin-biotin system*

Avidin is a basic glycoprotein (pI = 10.5) with a molecular weight of 68,000 daltons found in egg-white. It is capable of binding four biotin molecules with very high affinity (Ka = 10^{15} M^{-1}). Biotin, a naturally occurring vitamin with a molecular weight of 244 daltons can easily be coupled via a hydroxy succinimide derivative to proteins. The simple addition of avidin to biotinylated proteins forms the desired complexes. This technique is now widely employed in histochemistry and in ELISA techniques, for the binding of antibodies or lectins to enzymes or other marker proteins (Shamsuddin et al., 1983; Takai et al.,

1987; Plebani et al., 1984; Vilja et al., 1985; Lee et al., 1985; Lin et al., 1984; Guesdon et al., 1979; Bonnard et al., 1984; Henke et al., 1984; Wilchek et al., 1988).

The very high isoelectric point of avidin may result in non-specific binding due to electrostatic interactions to negatively charged groups such as sialic acids linked to glycoproteins or cell membranes (Glass et al., 1981; Bussolati et al., 1983; Banerjee et al., 1984; Hoffmann et al., 1980). In contrast to streptavidin, hen egg-white avidin is a glycoprotein, allowing the interaction with lectins binding to its glycans (Green et al., 1970). These disadvantages can be circumvented with the use of streptavidin, an extracellular product of Streptomyces avidinii. This protein is very similar to avidin, with a molecular weight of 60,000 dalton consisting of four identical subunits, each containing a single biotin-binding site. It has a near neutral isoelectric point (6.5) and it is free of carbohydrate side chains. During the course of this work streptavidin-peroxidase (STAP) became commercially available and it replaced the individual reagents avidin and peroxidase in the subsequent experiments.

The avidin-biotin amplified ELISA system was chosen in the second type of lectin-enzyme immunoassays for three reasons. Firstly, the strength of binding of lectins to carbohydrate groups (Ka = approx. 10^4 M^{-1}) is less than the antibody-antigen interaction (Ka = 10^7 M^{-1}). This may result in loss of bound lectin, for instance during washings of the tubes (Cerven et al., 1981). By complexing lectins in such a way that multiple interactions may take place, its was reasoned that this problem could be overcome. Indeed, no loss of binding was observed in our avidin-biotin based lectin-enzyme immunoassays even after many washings (chapter 3.3.11), while others report 15-20% loss after each washing (using ^{125}I-labeled CJA-lectin) (Cerven et al., 1981). Secondly, it was reasoned that simple conjugation of enzyme to lectin would provide insufficient signal. The fractions to be quantitated with our lectin-enzyme immunoassays may be very minor fractions of the glycoprotein; maximum signal amplification is thus necessary. The avidin-biotin system provides such an amplification by complexing a large number of biotinylated enzyme molecules with avidin or streptavidin, especially when using spacer arms (Hofmann et al., 1982; Leary et al., 1983). Thirdly, labeling of lectins with radioactive iodine results in alterations of binding characteristics (Montelaro et al., 1983; Armstrong et al., 1987). Such alterations have not been described for biotin-labeled lectins.

3.1.4. *The choice of horseradish peroxidase as the marker enzyme*

Horseradish peroxidase was chosen as the marker enzyme, as it compares favourably in ELISA techniques with other enzymes such as alkaline phosphatase or β-galactosidase (Porstman et al., 1985; Beyzavi et al., 1987; Nugel, 1986).

The experiments leading to the optimal concentrations of lectin, avidin and peroxidase are mentioned only briefly, as in the course of the experiments it was decided to use the preformed complexes of streptavidin and peroxidase

that came available from Amersham International. These complexes were mixed with biotinylated lectin one hour before use. In this way one incubation step (of preformed lectin-streptavidin-peroxidase complexes) replaces three subsequent incubations (of biotinylated lectin, followed by avidin, and followed by biotinylated peroxidase), leading to a substantial decrease of analysis time and variations of results.

During the initial development of lectin-enzyme immunoassays a number of difficulties was encountered. High blank values were observed after coating of microtiter plates with the IgG fraction of the antiserum, indicating binding of the lectin to the immobilised antibody in the absence of sample (results not shown). It was therefore decided to use affinity-purified antibodies to raise the "signal to noise ratio".

To establish the relation between the binding of a galactose-specific lectin in a lectin-enzyme immunoassay and the number of terminal galactose groups of the bound glycoprotein, we choose transferrin as the model compound, as discussed above. Most transferrin bears four sialic acid groups, two on each glycan. It was reasoned that by means of a short neuraminidase treatment the less sialylated glycovariants could be prepared, followed by purification by means of a high-resolution focusing or anion-exchange technique. This procedure would yield well-defined sialovariants, whose response in the lectin-enzyme immunoassay could subsequently be investigated.

A short incubation time with neuraminidase necessitates a fast removal (or inactivation) of the enzyme. Neuraminidase immobilised to Sepharose allows a quick removal (by centrifugation or column elution) of the glycoprotein in solution. By varying the incubation time one might expect to get differently sialylated transferrin variants. Still, each incubation time will probably lead to a mixture of sialovariants.

We preferred the development of Mono P and Mono Q column separation methods over other methods such as flat-bed isoelectric focussing in gels, because of the fast and preparative character of the column methods. Although the resolution obtained with isoelectric focussing, especially in immobilised pH gradients, is higher, the resolution of the Mono P and/or Mono Q technique was expected to be sufficient for this particular separation.

We have not considered the isolation and use of transferrin sialovariants present in human serum to be used as standards. First, the concentrations of the compounds other than 4-sialo transferrin are low, especially the extremely high and low sialylated sialovariants. Second, although their N-acetylneuraminic acid content may be deduced from their eluting position from Mono P or Mono Q columns, the terminal galactose expression is essentially unknown. Probably, even the sialovariants are complex mixtures, making them unsuitable for use as standards in the lectin-enzyme immunoassay. Moreover, if sulphate or phosphate is present, even the sialic acid content is uncertain.

3.2.1. *Materials*

The chemicals used were from Merck (Darmstadt, Germany) unless stated otherwise. OPD was obtained from Sigma (St. Louis, USA). RCA lectin and HRP (horseradish peroxidase) were from Boehringer (Mannheim, Germany). RCA-Agarose was from E.Y. Laboratories (San Mateo, USA). LCA-Sepharose and CNBr-activated Sepharose were from Pharmacia (Uppsala, Sweden). The proteins AAT, AGP, BG III, GC and TF and their antibodies were a gift from Behringwerke (Marburg, Germany). Goat-anti-Rabbit-IgG and its $F(ab')_2$ fragments were from Pel-Freez (Rogers, USA). N-hydroxysuccinimidobiotin, egg white avidin and pepsin were from Sigma. Streptavidin-biotinylated-peroxidase complexes were from Amersham Int. (Amersham, England). Polystyrene microtiter plates were obtained from Greiner (Langental, Switzerland). Antitransferrin was from Dako (Glostrup, Denmark).

Human transferrin was obtained from Sigma (St. Louis, USA), and saturated with iron in a bicarbonate solution at pH 8.0 for 24 hours. Bis-tris and bis-tris-propane were also from Sigma. Neuraminidase (from Clostridium perfringens) was obtained from Behringwerke (Marburg, Germany). CNBr-activated Sepharose 4B and polybuffer 74 were purchased from Pharmacia (Uppsala, Sweden). The chromatographic experiments were performed using a FPLC apparatus (Fast Protein Liquid Chromatography), also from Pharmacia.

3.2.2. *Methods*

Desialylation of transferrin

Neuraminidase (1 U) was coupled to CNBr-activated Sepharose 4B according to the procedures as described by the manufacturer. 2 ml of Sepharose-coupled neuraminidase was equilibrated with 50 mM sodium acetate buffer pH 5.5, containing 1 mM $CaCl_2$ and 150 mM NaCl. 10 mg iron-saturated transferrin in 250 μl of the same buffer was incubated for different lengths of time on the column and eluted with PBS, pH 7.4. The incubation times were 1 minute, 5 minutes, 1 hour and 20 hours at room temperature. The resulting fractions, containing transferrin in an increasing degree of desialylation, were then pooled in such a way that a "standard" mixture was obtained containing roughly equal amounts of the five sialovariants of transferrin.

Chromatofocusing on Mono P

The Mono P 5/20 column (0.5 cm diameter, 20 cm long) was equilibrated with starting buffer (1 ml/min of 25 mM bis-tris, pH 6.65, containing 10 mM NaCl and 3 μM $FeCl_3$). 500 μl "standard" mixture with a transferrin concentration of 1 mg/ml was applied to the column. After washing with starting buffer the pH-gradient was formed by elution with polybuffer 74 (diluted 1:10, containing 10 mM NaCl and 3 μM $FeCl_3$, pH 5.2). The pH of the eluting fractions was determined using a pH-meter.

Anion-exchange chromatography on Mono Q

100 μl of the "standard" mixture is brought onto the Mono Q 5/5 column (0.5 cm diameter, 5 cm long). The starting buffer was 20 mM bis-tris-propane, pH 7.0, containing 3 μM $FeCl_3$. The salt gradient was formed with 300 mM NaCl in starting buffer. Flow rate was 1 ml/min.

Carbohydrate analysis

Transferrin and asialo-transferrin were subjected to methanolysis (1.0 M methanolic HCl, 24 h at 85°C) followed by gas-liquid chromatography of the trimethylsilylated (N-reacetylated) methyl glycosides on a capillary CPsil5 WCOT fused silica column (0.34 mm x 25 m) (Chrompack, Middelburg, The Netherlands) (Kamerling et al., 1982).

Preparation of F $(ab')_2$ fragments

A few ml antibody solution was acidified with 1 M HCl to pH 3.8. Then 0.02 mg pepsin was dissolved per mg protein, and the mixture was incubated for 20 hours at 37°C. Any precipitate was removed by centrifugation, the mixture was made alkaline (pH = 8.0) with 1 M NaOH, followed by dialysis against 0.1 M NaCl containing 15 mM NaN_3.

Biotinylation

Antibody was diluted in PBS to a concentration of 10 mg/ml. A N-hydroxysuccinimido-biotin derivative was dissolved in DMSO to a concentration of 1 mg/ml. 250 μl antibody-solution and 250 μl biotin-DMSO solution were added to 750 μl of a 0.1 M carbonate-buffer pH 9.2 and 3.5 ml PBS. The reaction was allowed to take place for three hours at room temperature. Subsequently, the solution was dialysed for three days at 4°C against PBS with 6 buffer exchanges, or alternatively desalted on G-25 or PD-10 gel filtration columns.

Immobilisation of proteins on Sepharose

The immobilisation of proteins on CNBr-activated Sepharose was done as described by the manufacturer (Pharmacia, Uppsala, Sweden).

Affinity purification of antiserum

The antigen column was eluted until a stable base line was obtained, first

with PBS, then with 0.1 M glycine buffer pH 3.0 containing 1 M NaCl, followed by PBS. Sample was applied onto the column and allowed to interact for 30 minutes. All unbound proteins were eluted with PBS and discarded. The specific antibody was eluted with 0.1 M glycine buffer pH 2.8, and collected in 0.25 M veronal buffer pH 9.5.

Antibody coating of microtiterplates

Microtiter plates were coated with a predetermined dilution of antitransferrin or its F(ab')2 fragments just giving maximum results, for various lengths of time. Coating appeared to be complete within 10 minutes, but overnight coating was used as a routine.

3.3. Experiments and Results

The carbohydrate analysis of transferrin and asialo transferrin is given in table 3-1. It indicates complete removal of the four sialic acids by immobilised neuraminidase.

Table 3-1. Carbohydrate analysis of transferrin and asialotransferrin, expressed as mol /3 man

	Transferrin	Asialotransferrin
Man	3	3
Gal	2.0	1.7
GlcNAc	3.9	3.8
NeuAc	2.0	0

Figure 3-1A shows the Mono P elution patterns of the five human transferrin sialovariants prepared in vitro. The pH values of the eluting fractions are as follows: asialo 5.85, monosialo 5.70, disialo 5.54, trisialo 5.37 and tetrasialo 5.21. Figure 3-1B shows the Mono Q elution pattern of the same sialovariants.

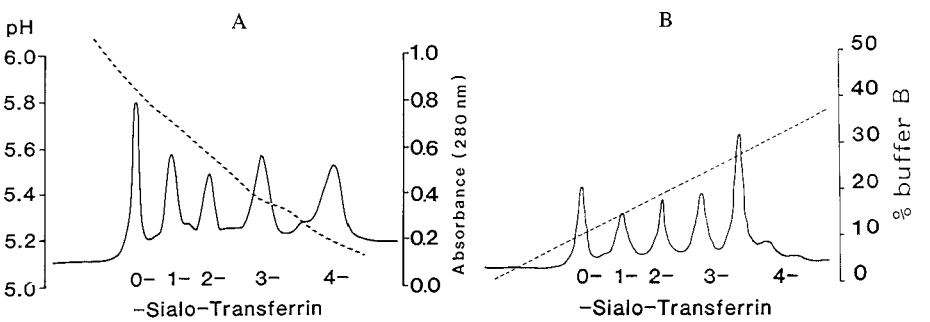


Figure 3-1. Separation of transferrin sialovariants on Mono P column (A) or Mono Q column (B).

The fractions elute at the following buffer B percentages: asialo 11, monosialo 15, disialo 19, trisialo 24 and tetrasialo 28.

3.3.1. *Determination of optimal avidin, lectin and peroxidase concentrations*

A number of experiments were done with subsequent incubations of RCA-biotin, avidin and peroxidase-biotin (always with four washings between each step) to determine the combination of concentrations that would give maximum enzyme activities. These experiments led to the following protocol:

1. Incubate antibody coated plates (50 μl, 16 h) with 50 μl sample for 2 hours.
2. Incubate with 50 μl RCA-biotin (20 μg/ml) for 1 hour.
3. Incubate with 50 μl avidin (10 μg/ml) for 1 hour.
4. Incubate with 50 μl peroxidase-biotin (7 μg/ml) for 1 hour.
5. Peroxidase reaction.

This protocol applied to transferrin samples gives a standard curve of asialo transferrin (figure 3-2).

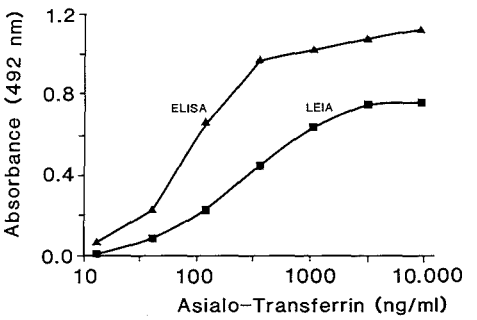


Figure 3-2. ELISA and LEIA standard curve of asialotransferrin.

3.3.2. *Premixing of reagents*

To diminish the large number of incubations and wash steps, it was decided to use preformed complexes by premixing the reagents. RCA-biotin and HRP-biotin were added to the microtiter wells, followed by a simple addition of avidin to complex the compounds. The following protocol was used:

1. Incubate antibody coated plates (50 μl, 16 h) with 100 μl asialo-transferrin for 2 hours.
2. Incubate with 30 μl RCA-biotin; 20, 10 or 5 μg/ml, and 30 μl HRP-biotin; 20, 4 or 1 μg/ml for 1.5 hours.
3. Add avidin; 60, 20, 6 or 2 μg/ml in 50 μl and allow to react for 1.5 hours.
4. Peroxidase reaction.

The results are shown in figures 3-3, 3-4 and 3-5 for 10 μg/ml RCA (the other RCA concentrations gave comparable results).

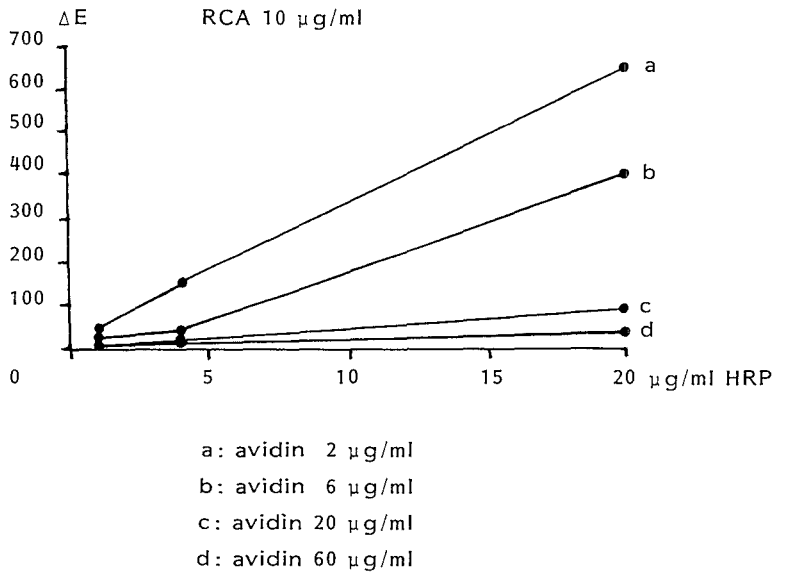


Figure 3-3.

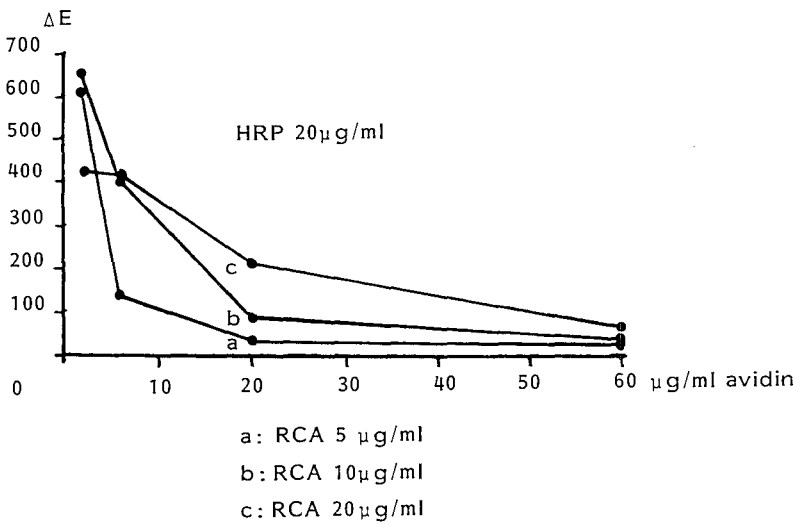


Figure 3-4.

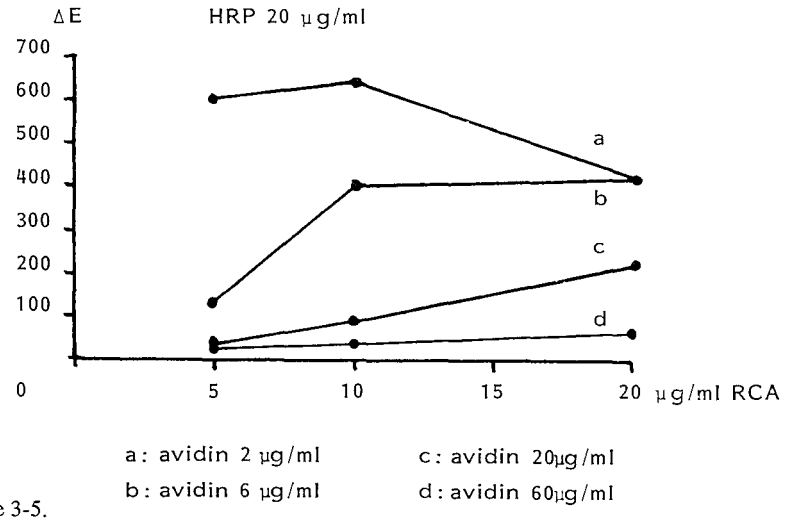


Figure 3-5.

3.3.3. *Premixing of reagents with lower concentrations of avidin*

1. Incubate antibody coated plates (50 µl, 16h) with 100 µl asialo-transferrin for 2 hours.
2. Incubate with 30 µl RCA-biotin; 20, 10 or 5 µg/ml, and 30 µl HRP-biotin; 20, 4 or 1 µg/ml for 1,5 hours.
3. Add 60 µl avidin; 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.8 µg/ml and incubate for 1.5 hours.
4. Peroxidase reaction.

Figures 3-6 and 3-7 show the results. A standard curve of asialo transferrin was produced with this method. The curve is presented in figure 3-8.

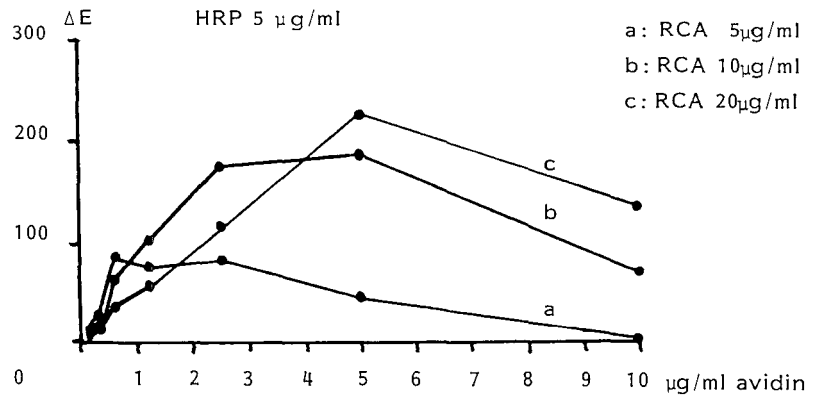


Figure 3-6.

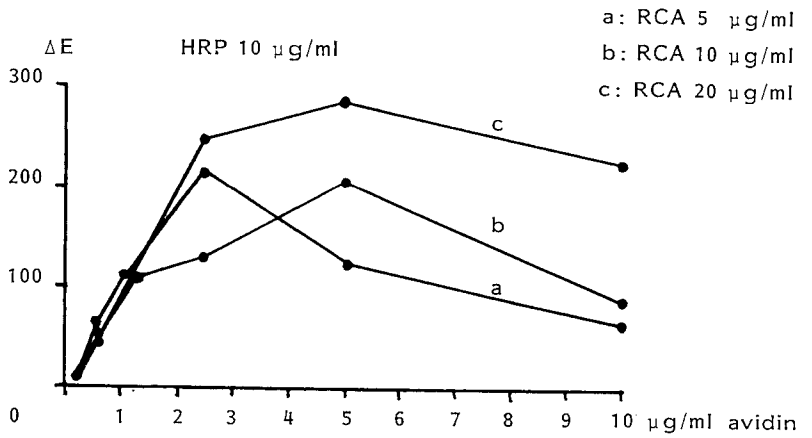


Figure 3-7.

Figures 3-3 through 3-7. RCA binding in LEIA without intermediate washings as a function of RCA, avidin and peroxidase concentrations.

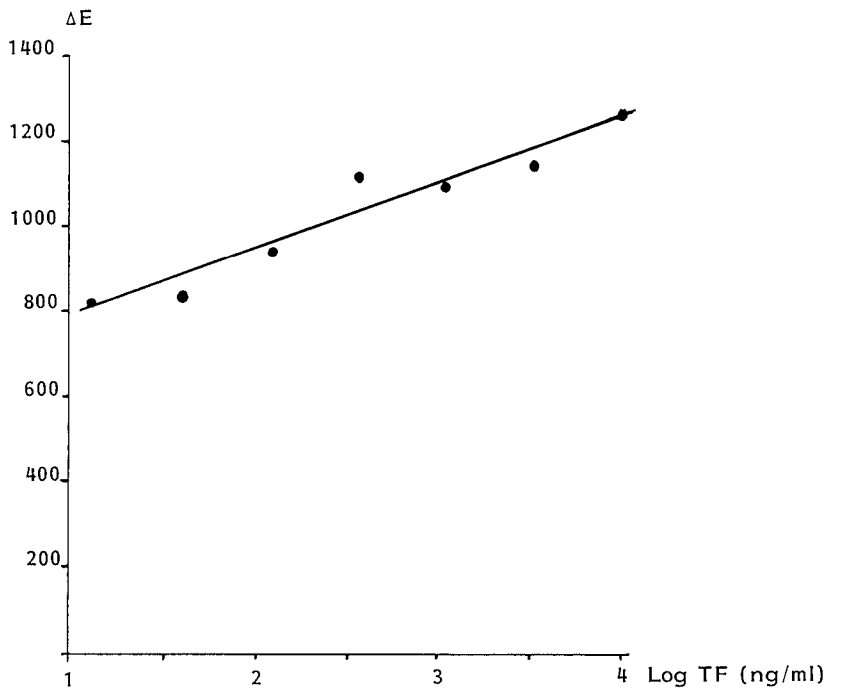


Figure 3-8. LEIA standard curve in the direct addition method.

3.3.4. *Influence of rheumatoid factor (RF)*

At this stage the performance of the lectin-enzyme immunoassay was challenged with plasma samples, the medium for which the assay was ultimately developed. Experiments with plasma samples from patients with rheumatoid arthritis indicated that very high results may be obtained, exceeding the highest standard, probably caused by IgM-RF binding to the coating and subsequent binding of RCA binding to IgM (Saltvedt et al., 1975). This was also shown using pre-immune IgG coated plates. RF also interferes in classical ELISA systems. It was decided to use $F(ab')_2$ coated wells, since most RF's bind to the Fc-fragments of immunoglobulins. The elimination of IgM-RF was checked using 2 microtiter plates coated with anti-transferrin and with $F(ab')_2$ anti-transferrin, respectively.

1. Coat with 50 μl anti-transferrin IgG or $F(ab')_2$.
2. Incubate with 100 μl plasma, diluted 1:100 with PBS for 2 hours.
3. Incubate with 50 μl diluted anti-IgM-peroxidase complex for 2 hours.
4. Peroxidase reaction.

Table 3-2 gives the results.

Table 3-2. Extinctions in LEIA with RA samples (R) and tumor samples (T) in wells coated with antitransferrin IgG (left) or $F(ab')_2$ (right).

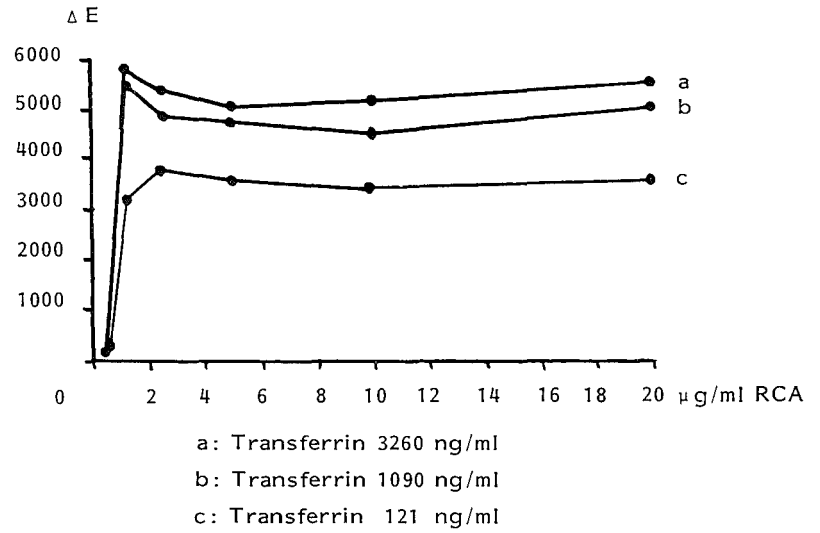
R 22	3151	31
R 23	2251	48
R 24	2086	153
R 25	2155	40
R 26	2713	109
R 27	1384	61
R 28	160	32
R 29	1636	42
R 30	652	20
T 5	434	79
T 6	379	63
T 17	121	36
T 21	1804	67

3.3.5. *Preformed lectin-streptavidin-peroxidase complexes*

The availability of stable complexes of streptavidin with peroxidase-biotin (STAP) (Amersham) opened the way of reproducible premixing of reagents. The minimal RCA-biotin concentration was determined as follows.

1. Coat with $F(ab')_2$ anti-transferrin (50 μl, 16 h).
2. Incubate with 50 μl asialo-transferrin for 2 hours.
3. Incubate with a 1 hour premix of STAP diluted 1:1250 or more and RCA-biotin; 0.32 - 20 μg/ml.

Results are represented by figures 3-9 and 3-10.



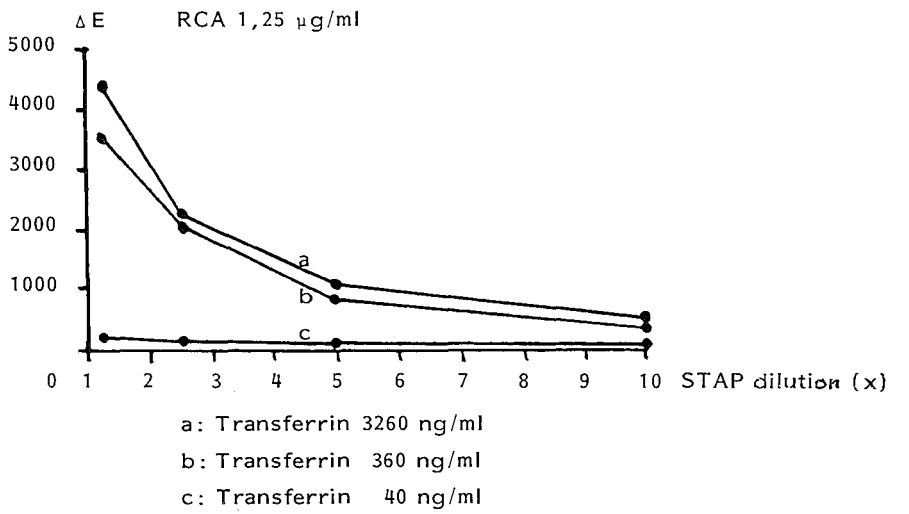
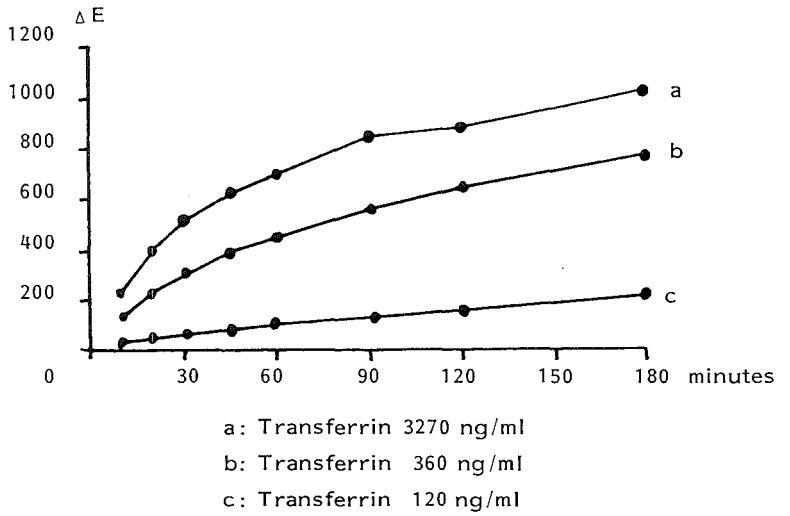


Figure 3-9 (top) and 3-10. LEIA response as a function of RCA and STAP concentrations.

3.3.6. *Influence of incubation time*

The binding of RCA-STAP complex as a function of the incubation time was investigated as in 3.3.5. Incubation times ranged from 10 to 180 minutes. RCA: 1 µg/ml, STAP 1:2500. Figure 3-11 shows the result.



3.3.7. *Influence of preincubation time*

To determine the length of time needed to form complexes between RCA and STAP, the pre-mixing time was investigated between 15 and 120 minutes. The extinctions measured are shown in figure 3-12. Reaction conditions as in 3.3.6.

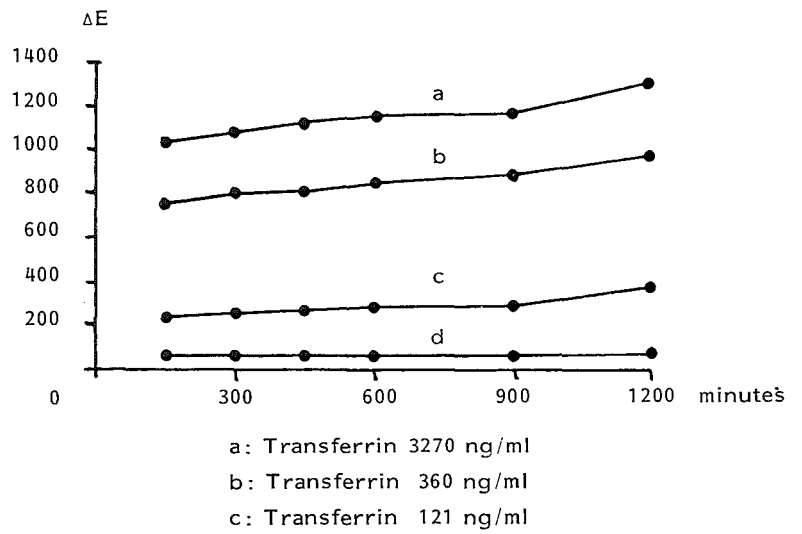


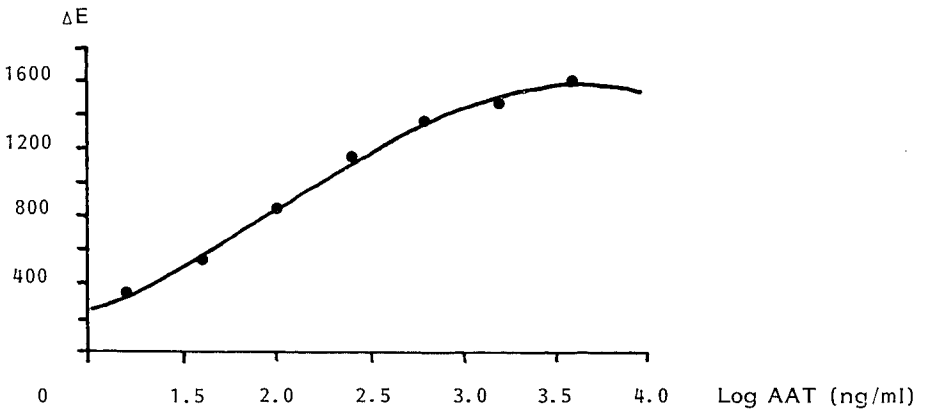
Figure 3-12. LEIA response as a function of RCA /STAP preincubation time.

3.3.8. *Influence of the number of washings*

1. Coat with 50 μl F(ab')$_2$ anti-transferrin for 16 hours.
2. Incubate with 50 μl asialo transferrin for 2 hours.
3. Incubate with an 1 hour premix of 1 μg/ml RCA-biotin and STAP 1:2500 for 1.5 hours.
4. Wash x times (0-6).
5. Peroxidase reaction.

3.3.9. *Standard curves of asialo* α_1-antitrypsin, β_2-glycoprotein III and Gc-globulin.

Microtiterplates were coated with F(ab')$_2$ fragments of affinity purified antibodies against these proteins (50 μl, 16 h). Other reaction conditions as in 5.3.8. The standard curves obtained with α_1-antitrypsin and β_2-glycoprotein III are given in figures 3-13 and 3-14.



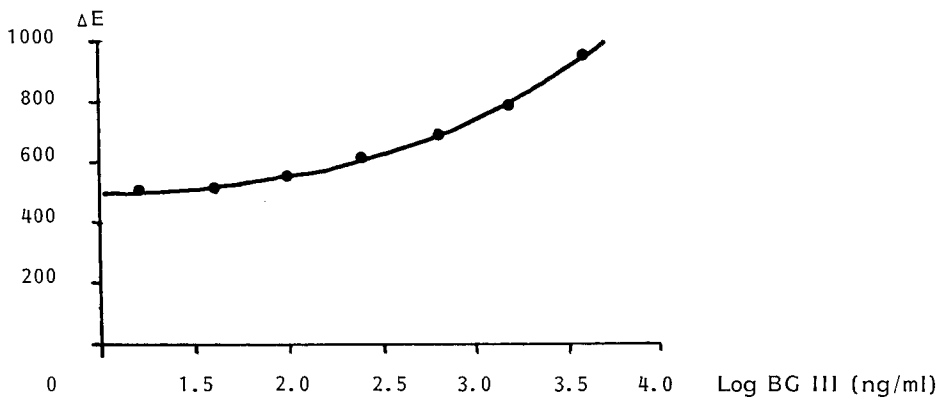


Figure 3-13 (top) and 3-14 (bottom). LEIA standard curves with RCA for ATT and BGIII.

3.3.10. *Response of transferrin sialovariants*

Transferrin sialovariants were prepared and separated as described in chapter 4. They were diluted to a concentration of 10 μg /ml before incubation in $F(ab')_2$ coated plates. Other reaction conditions as in experiment 3.3.8. The response of the five sialovariants is given in figure 3-15.

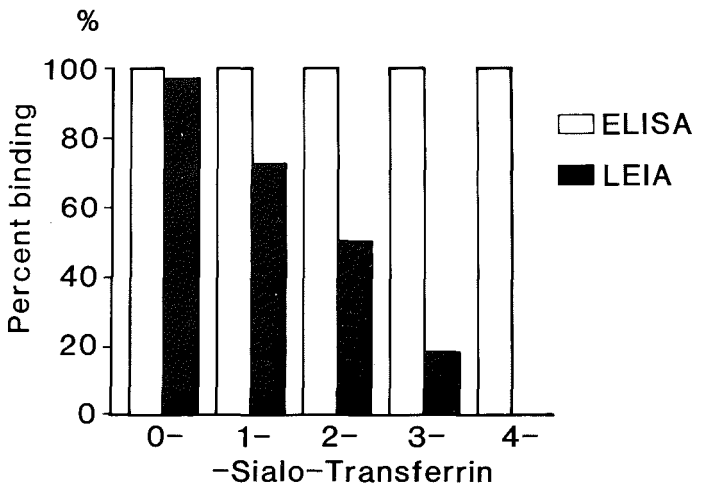


Figure 3-15. ELISA of five transferrin sialovariants and LEIA response with RCA lectin.

3.3.11. *Influence of the number of washing steps on RCA-STAP binding to the various transferrin sialovariants*

Plates were rinsed 3, 4, 5, and 6 times to detect any galactose-density dependent loss of RCA-STAP binding. Experimental conditions as in 3.3.8. Figure 3-16 shows the results.

3.3.12. *Dilution of transferrin sialovariants*

To investigate the influence of surface galactose density on the binding of lectin-STAP complex, an experiment was done with dilutions of the $F(ab')_2$ coating. If the surface galactose density has a different influence on the binding of RCA to each of the transferrin sialovariants, this experiment should show it. Coating: $F(ab')_2$ fragments, 50 μl, 16 h, diluted 2, 4, 8, 16, 32 and 64 times. Other reaction conditions as in 3.3.8. Figure 3-17 shows the response of the five sialovariants.

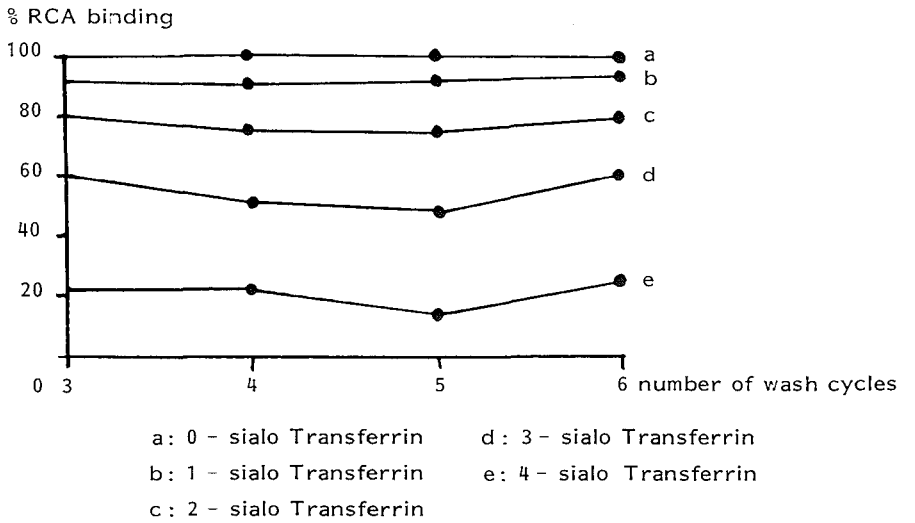


Figure 3-16. Influence of wash cycles on LEIA response as a function of terminal galactose groups.

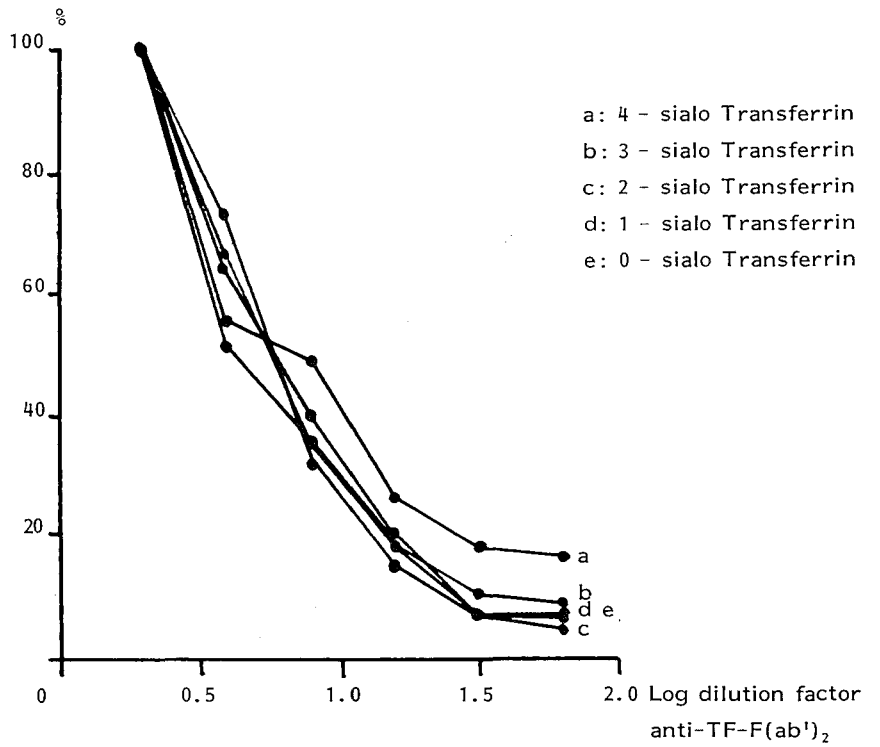


Figure 3-17. Effect of diluting the coating antibody on the results of the lectin-enzyme immunoassay for the five transferrin sialovariants.

3.3.13. *Response of LCA-binding transferrin in the lectin-enzyme immunoassay*

The standard curve is shown in figure 3-18. The influence of the LCA concentration on the response of the various standards is given by figure 3-19.

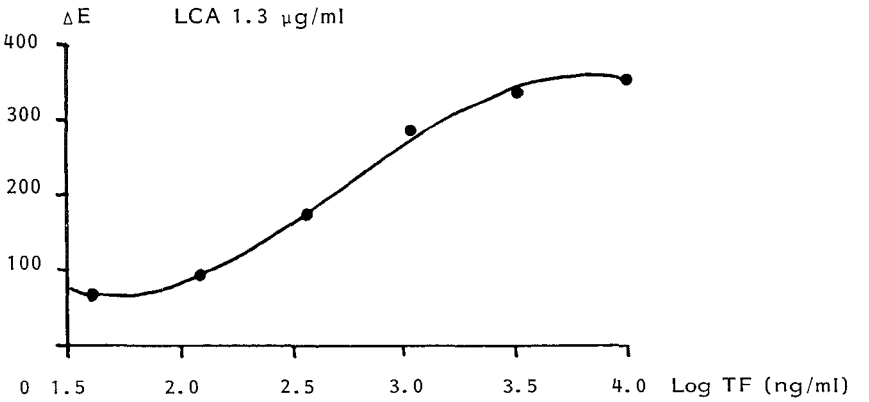


Figure 3-18. LEIA standard curve of LCA binding transferrin.

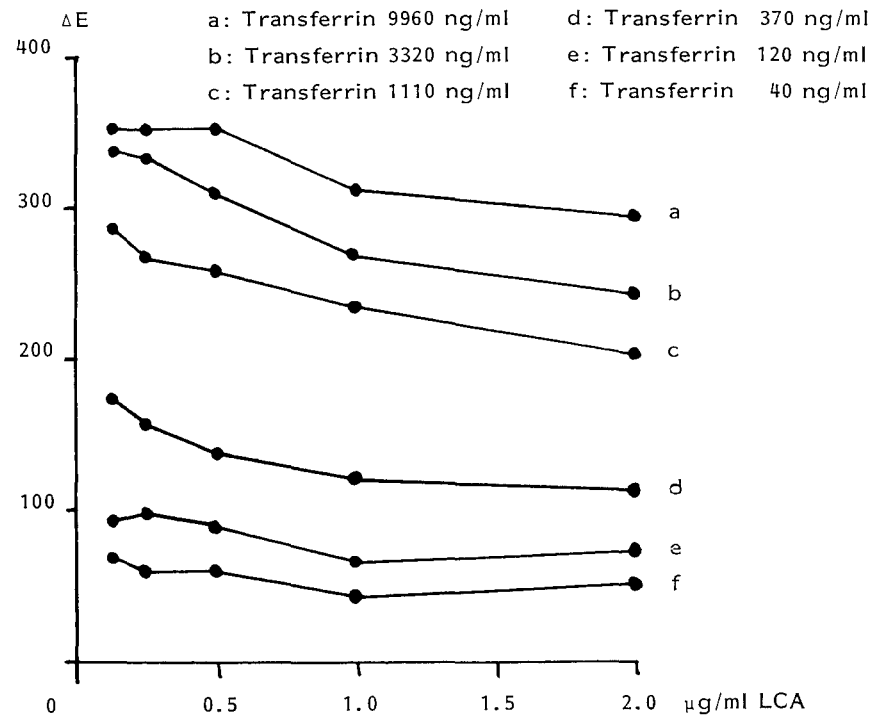


Figure 3-19. LEIA response as a function of LCA concentration.

3.4. Overview and discussion of the results

As can be seen in figure 3-1A and figure 3-1B the resolving power of Mono P and Mono Q are roughly equal. The latter technique however, has a few distinct advantages. Firstly, the anion-exchange chromatography takes considerably less time. Secondly, as no ampholytes are involved, the Mono Q separation produces pure transferrin sialovariants ready for use in other experiments. Thirdly, the pH used in the Mono Q separation bears less risk of sialic acid loss from the glycan. Most sialic acid groups are sensitive to hydrolysis in an acid environment. And fourthly, the Mono Q separation is cheaper as it employs no ampholytes.

It is unknown why the separation of transferrin sialovariants can be obtained (either with Mono P or Mono Q), while most other glycoprotein sialovariants elute much closer to each other, not allowing complete separation. Possibly the distribution of the glycans on the surface of the molecule is of importance.

The stepwise incubations of asialo transferrin immobilised to a solid phase with biotinylated RCA, avidin and biotinylated peroxidase (experiment 3.3.1) indicate that in principle this approach is suitable to detect asialo transferrin in a lectin-enzyme immunoassay (fig. 3-2). However, large variations between individual results were obtained (not shown), suggesting that the number of incubations and wash steps (twelve in all) should be decreased.
This could be achieved by adding simultaneously biotinylated RCA and biotinylated peroxidase, followed without washing by the addition of avidin which forms the desired complexes (experiments 3.3.2. and 3.3.3.).

As experiment 3.3.2 shows (figures 3-3, 3-4 and 3-5) this approach also works well, as long as the HRP-biotin concentration is above 20 μg/ml. Lower HRP concentrations lead to very low extinctions. The avidin concentrations used were considered to be too high, given the response shown by figure 3-4. In a second experiment (3.3.3) avidin concentrations between 10 and 0.8 μg/ml were used. Figures 3-6 and 3-7 show the dose-response curves for 5 and 10 μg/ml HRP, respectively, and for 5, 10 and 20 μg/ml RCA as a function of the avidin concentration. The avidin concentration of 5 μg/ml and the RCA concentration of 20 μg/ml were suitable for further use. The amount of HRP is not critical: more HRP leads to higher extinctions.
Figure 3-8 shows the dose-response curve with this method as a function of the asialo transferrin concentration. The very low slope indicates that the method in this form is not suitable for use in a lectin-enzyme immunoassay.

Another problem appeared to be the high background signal caused by rheumatoid factors. These antibodies to IgG may bind to the coated IgG. If they are of the IgG_3, IgM or IgA class, they may bind RCA. Most RF's are IgM. Thus, serum samples containing RF will show falsely elevated values in the lectin-enzyme immunoassay for galacto-transferrin, even without any transferrin present. Experiment 3.3.4 indicates the interference from RF's (table 3-2). Use of $F(ab')_2$ coating is recommended.

At this stage, the preformed streptavidin-biotinylated peroxidase (STAP) complexes became commercially available. When biotinylated lectin is mixed with STAP before use, only one incubation step is needed for the three components. Besides, the premixing leads to multiple lectins complexed to each other, which may be advantageous in view of the low association constant of most lectins. The advantages of streptavidin over avidin (its neutral pI (6.5 vs 10.5) and its lack of carbohydrate side chains) are of less importance here.

To determine the best proportions of RCA and STAP experiment 3.3.5 was conducted. Any RCA concentration above 1 μg/ml worked well (figure 3-9). A STAP dilution experiment showed that dilutions further than 1:2500 lead to low absorbance values (figure 3-10).

In chapter 4 a new fast method is described making use of avidin coated plates to find the optimal lectin-streptavidin-peroxidase complex composition without using the lectin binding function.

As the reagents are premixed, complex formation may take place during the incubation steps. This was investigated in experiment 3.3.6. As can be seen in figure 3-11 the measured extinction continues to rise for at least three hours. 90 minutes was arbitrarily chosen for the subsequent experiments.

Experiment 3.3.7 (figure 3-12) indicates that complex formation occurs within 15 minutes after premixing of RCA-biotin and STAP. We selected a pre-incubation time of 60 minutes, but this is not critical.

The relatively low binding constant of lectin-glycan interaction might cause loss of bound lectin during the washing steps. This has been observed for ^{125}I-labeled Crotalaria juncea lectin (CJA) binding to galacto-transferrin (Cerven et al., 1981) where each washing induces a loss of signal of approx. 20%. In that system, however, lectins were not complexed with STAP. The use of lectin-STAP complexes may reduce the liberation of lectin bound to the captured glycoprotein. No dependence was found in our system on the number of wash steps, indicating that the lectin-STAP complex is firmly bound to the captured transferrin having four terminal galactose groups (experiment 3.3.8.; results not shown) or less (figure 3-16). The antibody-antigen interaction too is apparently not disrupted.

It is concluded that lectins complexed with peroxidase by the avidin-biotin system and bound to the captured glycoprotein are not dissolved again by the washing procedure, in contrast to uncomplexed lectin. This finding supports the choice of the avidin-biotin system versus direct enzyme-conjugated or radio-iodinated lectin.

The experiments described in paragraph 3.3.9 indicate that the concept of lectin-enzyme immunoassay may also work for galacto-α1-antitrypsin (figure 3-13), but not (with RCA as the galactose specific lectin) for BG III (figure 3-14) or Gc (not shown). The reason for this discrepancy is not known. AAT contains three N-linked glycans but the structures of BG III and Gc are not known. In any case they contain only little carbohydrate (table 1-1). If they contain O-linked chains to which RCA does not bind, or if their structures are

resistant to neuraminidase these observations could be explained. Neuraminidase-treated BG III is not precipitated by RCA but Gc-globulin is (Uhlenbruck et al., 1978). On the other hand, N-acetylgalactosamine has been demonstrated in BG III but not in Gc-globulin, while Gc-globulin does not contain sialic acids while BG III does (Uhlenbruck et al., 1979).

The response of the five purified transferrin sialovariants in the lectin-enzyme immunoassay (experiment 3.3.10) is given in figure 3-15.

Two conclusions may be drawn. First, it is noted that the number of sialic acid groups does not have any influence on the response of the ELISA. The amount of each sialovariant bound to the coated antibodies is thus the same. Second, the RCA-STAP binding was found to be dependent on the number of galactose groups present on the transferrin molecule.

As the strength of binding to RCA lectin depends on the number of terminal galactose groups (Kornfeld et al., 1981; Baenziger et al., 1979), it could be reasoned that a preferential loss during wash cycles may occur of RCA-STAP complex from the higher sialylated transferrin sialovariants. The experiment described in 3.3.11. indicates that no liberation occurs from any of the transferrin sialovariants (figure 3-16). If lectin-STAP complexes are preferentially washed away from those glycoprotein glycovariants having less carbohydrate structures recognised by the lectin, the concept of lectin-enzyme binding assay would be without a firm basis.

The experiments described in paragraph 3.3.12 were performed to investigate the influence of the coating density on the binding of complexed lectins. It could be argued that the lectin-enzyme binding is a function of the galactose density in the coated microtiter plates. Figure 3-17 shows that this is not the case. The parallel lines indicate that the response remains a function of the number of terminal galactose groups per transferrin molecule, whatever the coating density.

As figure 3-17 shows, the density of the coating antibody does not influence preferentially any of the sialovariants. This may mean that the average coating distance between antibodies is larger than the average lectin-STAP complex.

To investigate the application of another lectin in the lectin-enzyme immunoassay we investigated the binding of the lectin from Lens culinaris (LCA), known to bind α1-6 fucosylated diantennary glycans (experiment 3.3.13). To obtain transferrin that binds to the lectin in the assay, a column of immobilised LCA was used to extract the binding fraction from commercially available transferrin. This approach is essentially different from the RCA binding fractions, where enzymatic (i.e. neuraminidase) treatment of the glycoprotein yields the standard material. In the case of LCA, and possibly a number of other lectins as well, the standard material must be isolated from the purified glycoprotein by lectin column chromatography using the same lectin as in the lectin-enzyme immunoassay. The standard curve obtained with the LCA binding transferrin thus isolated is shown in figure 3-18. Variation of the LCA-biotin concentration affected the results only slightly (figure 3-19). The same has been found for RCA-biotin. The LCA non-binding fraction, diluted to 10 μg/ml, did not show binding of LCA-STAP complex in the assay.

3.5. Discussion

The lectin-enzyme immunoassay described here is a new tool to investigate protein glycosylation and its modifications. It has a number of distinct advantages, such as speed, sensitivity, specificity and simplicity, and when using microtiter plates it is easily automated. In theory, purification of the glycoprotein being studied is not necessary. Since the assay was developed with purified transferrin sialovariants, it remains to be established to which extend the presence of other glycoproteins has an influence on the analysis.

In general, the standards used in the LEIA differ from those used in the ELISA, as they consist of subfractions of a glycoprotein. This means that enzymatic removal of sugar groups may be necessary to obtain the standard. We used neuraminidase to prepare the asialo-transferrin standard. In other cases (i.e. LCA-reactive glycoprotein) the standard material must be purified by isolating the subfraction in question with an immobilized lectin, the same as that used in the LEIA. The material probably does not have to be very pure with respect to contamination with other glycoproteins, but it must be pure with respect to its glycosylation.

The standard curve of the assay was constructed with compounds bearing whole integers of galactose groups. The result of an unknown sample, however, may lead to fractions; the average of a large number of differently galactosylated molecules. This means that the unit of expression of the lectin-enzyme immunoassay should be "average molar number of terminal galactose units".

The sensitivity of the LEIA has to be high when the subfractions to be quantitated make up only a small portion of the glycoprotein. This was achieved by two measures. Firstly, antibodies that were affinity purified over an immobilized antigen column were used as the coating material. This will increase the lectin binding to the captured glycoprotein by increasing the density of the coated specific immunoglobulin. Secondly, we used biotin-labeled lectin bridged by streptavidin to biotin-labeled peroxidase to increase the number of marker enzyme molecules bound per lectin molecule. This amplification system also increases the sensitivity of the LEIA. It also increases the binding affinity of the complexed lectin molecules to the captured glycoprotein molecules. In contrast, by using single lectin molecules one may observe loss of binding during each washing step (Cerven et al., 1981). This was not observed in our assay.

Recently three enzyme-linked and one radio-labeled lectin binding assay have been described (Molin et al., 1986; Van der Schaal et al., 1984; McCoy et al., 1983; Cerven et al., 1981) Our assay is distinct in several respects. Both the enzyme-linked lectin binding assay (ELBA) described by Van der Schaal et al. and the enzyme-linked lectin assay (ELLA) described by McCoy et al. used passive adsorption of a glycoprotein to polystyrene. The ELBA method was used to determine the lectin affinity of the Pea lectin by inhibition of the binding by mono- and polysaccharides. The ELLA method was used to study the expression of α-D-galactopyranosyl end groups of natural and synthetic glycoproteins using the Bandeira (Griffonia) simplicifolia I B4 isolectin. These methods work well with pure glycoproteins or glycolipids.

The binding of lectins to glycosphingolipids was investigated in a lectin-enzyme binding assay (Molin et al., 1986). The lipids were adsorbed in microtiter plates by evaporation of the methanol solvent from the cholesterol, lecithin and lipid mixture. Biotinylated lectins were allowed to bind, and after washing, a solution of avidin-peroxidase conjugate was added. With this system, the affinity of the lectins PNA, WGA and UEA-I for a number of lipids was characterised.

In our assay an antibody-coated solid phase was used. This has the advantage of specificity with respect to the glycoprotein under study when it is present in a complex mixture. Passive adsorption can be used only when purified glycoprotein is available. However, not all proteins bind very well to polystyrene surfaces. Because immunoglobulin G binds strongly, such proteins can also be analysed if antibodies are available.

The radiolectin immunoassay technique (RLIA) described by Cerven et al., was used to study the glycosylation of transferrin sialovariants with a ^{125}I-labeled galactose-binding lectin isolated from Crotalaria juncea seeds and with the ^{125}I-labeled sialic acid-binding lectin from Limulus polyphemus. CNBr-Sepharose-bound anti-transferrin served as the solid phase. Increased desialotransferrin (pI 5.7) concentrations were found in serum from alcoholics, in agreement with other reports (Stibler et al., 1981; 1986). However, loss of binding occurred due to the relatively weak lectin-glycan interaction.

An alternative immunoassay was subsequently developed by the authors to avoid the problems associated with the loss of bound radiolabeled lectin during wash steps. They investigated the feasability of radiolabeling the sialic acid groups of transferrin bound by the immunoadsorbent, by oxidation with sodium metaperiodate followed by introduction of the label with sodium borotritide. The method exhibited high background values due to binding of the tritium label to the immunoadsorbent gel and to other sugars then sialic acid. Moreover, measurement of a small decrease in sialic acid content of transferrin such as occurs in alcoholics is quite difficult to quantitate in this way. The labeling immunoassay might have certain potentials if the terminal galactose groups were labeled (for instance with galactose oxidase followed by the sodium borotritide reductive labeling), as terminal galactose groups are sparse on human serotransferrin.

Our lectin-enzyme immunoassay (LEIA) has a number of advantages in comparison with the RLIA procedure. It uses the RCA lectin, which has been shown to bind to asioalotransferrin (Uhlenbruck et al., 1978) but which does not bind to immunoglobulin G except for the minor fraction IgG_3 (Saltvedt et al., 1975). It does not use ^{125}I-labeled lectins, which are shown to have altered carbohydrate-binding properties (Shimoda et al., 1985). The binding of the lectin is independent of the number of washing steps, while in the RLIA procedure 15-20% loss occurs during each wash cycle, probably due to a lower binding constant of single ^{125}I-CJA lectin molecules compared with biotin-streptavidin complexed RCA lectin molecules.

A theoretical disadvantage of our LEIA technique, i.e., the binding of the

lectin to the antibody coating, may occur. The use of affinity-purified antibodies increases the density of the captured glycoprotein and the lectin binding to it, probably without having an influence on the "aspecific" binding of lectin to the immunoglobulin coating ("aspecific" is written in quotation marks because of course the lectin binding to the immunoglobulin glycans is carbohydrate specific, but to the wrong molecules). The use of the $F(ab')_2$ fraction may further lower the background binding because most immunoglobulin glycans are Fc-linked (Taniguchi et al., 1985). If necessary, other measures can be taken to suppress background binding, such as affinity purification of the immunoglobulins on a column of an immobilized lectin (the same one as used in the LEIA). Although RCA column chromatography of the coating antibodies does increase the "signal to noise ratio" it does not completely eliminate the binding of enzyme-labeled RCA lectin to the coated immunoglobulins. Complete elimination of this background binding can probably only be achieved by removal of the Ig glycans by N-glycanase treatment, or by removal of galactose groups by sequential neuraminidase and β-galactosidase treatment. Preliminary experiments to reach this goal proved to be unsuccessful, probably due to the resistance of RCA binding Ig glycans to N-glycanase and galactosidase (data not shown). The right conditions to prepare aglyco- or agalacto immunoglobulins remain to be established.

Unexpectedly, the LEIA standard curve of the number of terminal galactose units plotted against the percentage of maximum enzyme activity bound consistently produces a straight line. As the form of the curve is determined by binding constants, size of the RCA-complexes, glycoprotein glycan exposition, etc. such a straight line is not necessarily expected.

With LEIA technique it is possible to study the glycosylation of very small amounts of a glycoprotein. The amount of carbohydrate present per well (calculated from the saturating amount of transferrin (200 ng) and given the 5% carbohydrate in transferrin) is approx. 10 nanograms. This indicates a very high sensitivity compared with other techniques.

One application is the investigation of the glycosylation of small amounts of glycoproteins produced by recombinant techniques and tissue cultures. Another is the change in plasma glycoprotein glycosylation in disease.

3.6. Conclusions

The experiments described in this chapter have led to a protocol for lectin-enzyme immunoassays. Although it was developed for the RCA lectin, the successful application of the LCA lectin shows that it is also applicable for other biotinylated lectins.

Methods using stepwise addition of lectin, avidin and enzyme are considered inferior compared with the one-step addition of premixed and complexed lectin-streptavidin peroxidase. Binding stability increases, and the number of incubation steps involved is reduced.

CHAPTER 4

LECTIN-ENZYME BINDING ASSAYS FOR THE CHARACTERISATION OF THE LECTIN SPECIFICITY AND FOR THE DETERMINATION OF EQUIVALENCY BETWEEN LECTIN-BIOTIN AND STREPTAVIDIN-PEROXIDASE

4.1. Introduction

It is at present not possible to predict which lectin is most suitable to detect disease-associated changes of a certain plasma glycoprotein. Increased branching, increased or absent sialylation, decreased galactosylation and increased fucosylation have all been described (Endo et al., 1987; Pekelharing et al., 1988; Thompson et al., 1987). On the other hand, the detailed binding specificities of most lectins have not yet been elucidated. Thus, both the glycan changes in disease of most plasma glycoproteins and the specificity of our "tools" remain obscure. This means that to find a fruitful combination of lectin and glycoprotein, a trial and error approach should be taken. Thus, a fast way to prepare lectin-STAP complexes without using the lectin binding function is a necessity. This chapter describes such a method.

The lectin-enzyme binding assay may be used to gain insight in the carbohydrate specificity of lectins. Inhibition studies using simple sugars as well as binding studies to immobilised neoglycoconjugates will lead to a more detailed description of the carbohydrate structures for which the lectin has affinity.

To be able to detect small subfractions of a glycoprotein, the lectin should not bind to the glycoprotein from healthy persons plasma. This may also be investigated by lectin-enzyme binding studies, using immobilised purified plasma glycoproteins.

4.2.1. *A lectin-enzyme binding inhibition assay in glycoprotein-coated microtiter plates to determine the lectin binding specificity*

Since the early days of 1888 when Stillmark discovered the "phytohemagglutinins", lectins are detected by their capacity to agglutinate red blood cells. Watkins and Morgan (1952) found that simple sugars are capable of inhibiting the hemagglutination. The hemagglutination inhibition system to investigate the lectin specificity has the advantage of being sensitive and very easy to perform: the hemagglutination is readily visualized in a test tube. On the other hand, the red blood cells contain a very large variety of structurally

different glycoproteins and glycolipids, each contributing to a different extent to the binding of the lectin used. Quantitation of the observed agglutination is also a problem.

A more defined system would be the inhibition by simple sugars of the binding of the lectin to a purified glycoprotein with well-known glycan structures. In such an assay, however, no red cells are available to serve as the detector system. The use of radioactive groups introduced in the lectin molecules would be a possibility, but ^{125}I-labeling may change the binding characteristics of lectins (Montelaro et al., 1983, Shimoda et al., 1985). Biotin labeling has the advantage of being relatively inert. The amount of bound biotinylated lectin may be quantitated using enzyme-labeled (strept-)avidin.

In the method that we developed we used a glycoprotein coated to a solid phase provided by a microtiter plate, to which the lectin-streptavidin-peroxidase complex may bind. The glycoprotein used is asialo transferrin, the lectin is Ricinus communis agglutinin I (RCA) and the enzyme is horseradish peroxidase. By inhibiting the binding of the lectin-enzyme complex to immobilized asialo transferrin with a number of simple sugars in various dilutions the lectin specificity is characterised.

4.2.2. *Materials and Methods*

All experiments were performed in 96 well microtiter plates from Greiner (Langental, Switzerland). Ricinus communis 120 lectin was obtained from Boehringer (Mannheim, Germany). Asialotransferrin was prepared by incubating 10 mg transferrin (Kabi, Stockholm, Sweden) for 24 hours at room temperature in 0.1 M acetate buffer, pH 6.8, containing 10 mM Ca^{2+} with 2 ml CNBr-activated agarose gel to which 1 U neuraminidase (Boehringer) has been covalently linked. Transferrin concentrations were determined by radial immunodiffusion. Streptavidin-biotinylated peroxidase complexes were obtained from Amersham (Amersham, England). Ricinus communis lectin was biotinylated by dissolving 10 mg of the lectin in 1.6 ml PBS buffer to which is added 1 mg N-hydroxysuccinimidobiotin (Sigma, St. Louis, USA), dissolved in 1 ml dimethylsulfoxide and 3 ml 0.2 M carbonate buffer, pH 9.2. After 3 hours incubation at 25°C the reaction mixture is dialyzed against 0.9% NaCl. The NaCl solution is changed six times. The biotinylated lectin is stored in 0.5 ml portions at -20°C. The microtiter plates were coated with asialotransferrin by incubating each well with 50 μl asialotransferrin in PBS (10 μg/ml) at 4°C.

After 16 h the plates were washed four times with PBS pH 7.4, containing 0.1% Tween 20. The wells were incubated at 25°C with 100 μl PBS-Tween, containing the competing sugar, Ricinus communis lectin (1.25 μg/ml) and the streptavidin peroxidase complex in a final dilution of 1:2500. After 70 minutes the plates were washed four times and the peroxidase activity was measured by incubating the wells with 100 μl 0.08 M Na_2HPO_4 /citric-acid buffer with 4.4 mM o-phenylenediamine and 3.5 mM H_2O_2. After 14 minutes the reaction

was stopped with 50 μl 2 M H_2SO_4. The absorbance was measured at 492 nm with a Titertek Multiskan Elisa reader.

4.2.3. *Results*

The kinetics of the adsorbance of asialo-transferrin coating to the plastic cuvettes of the microtiter plate shown in figure 4-1. We used overnight coating at 4°C as a routine, although 10 minutes at room temperature is sufficient. Figure 4-2 shows the increase with time in the binding of the lectin-streptavidin-enzyme complex to the coated asialo-transferrin. After three hours 95% of the maximum signal was reached.

The inhibition by various sugars of RCA binding to asialo-transferrin is shown in figure 4-3. The 50% inhibition concentrations for the various sugars are given in table 4-1A.

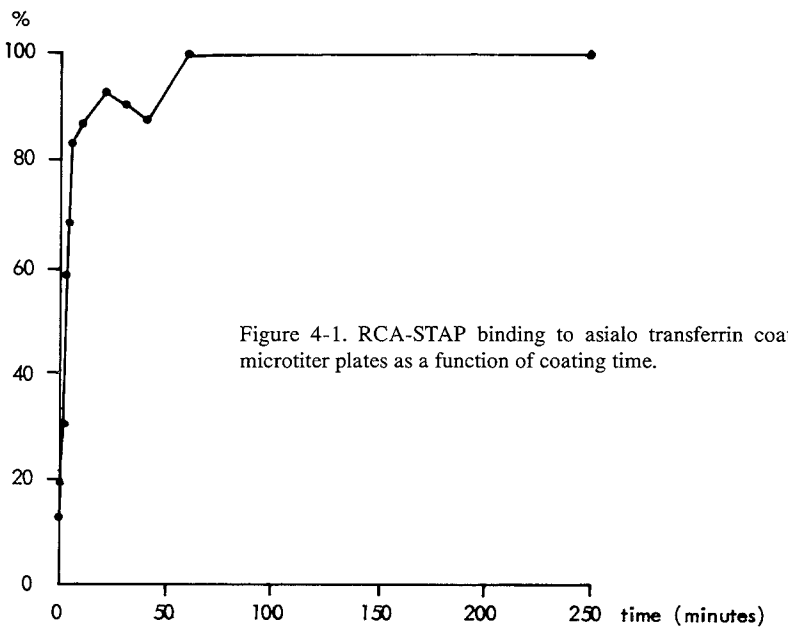


Figure 4-1. RCA-STAP binding to asialo transferrin coated microtiter plates as a function of coating time.

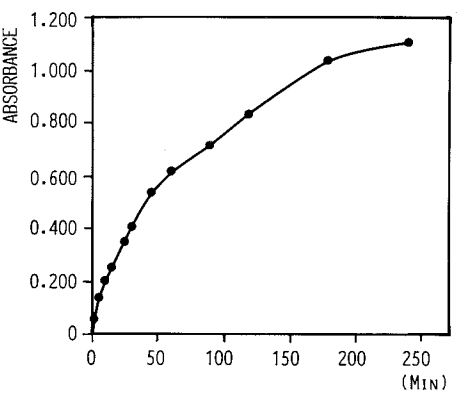


Figure 4-2. RCA-STAP binding to asialo transferrin coated microtiter plates as a function of RCA-STAP incubation time.

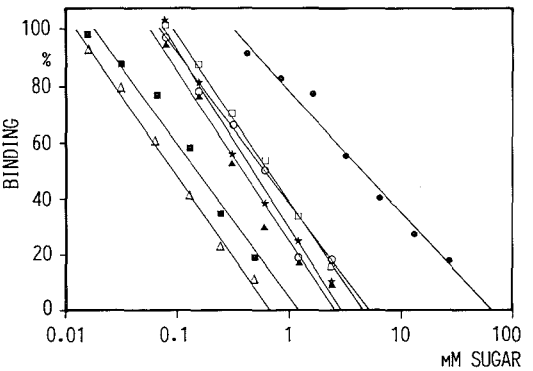


Figure 4-3. Inhibition by simple sugars of RCA-STAP binding to immobilized asialo transferrin.

Table 4-1A. 50% inhibition concentrations of RCA-STAP binding to immobilized asialo transferrin.

Sugar	50% inhibition concentration
△ ONP-β-D-galactose	0.09 mM
■ β-D-lactose	0.15 mM
▲ ONP-α-D-galactose	0.39 mM
✸ α-D-stachyose	0.46 mM
○ D-galactose	0.61 mM
□ α-D-melibiose	0.68 mM
● D-galactosamine	4.7 mM
Glucose	> 200 mM
Mannose	> 200 mM
Fucose	> 200 mM

4.2.4. *Discussion and Conclusions*

The results of hemagglutination inhibition tests are often expressed in terms of "relative inhibitory potency" arbitrarily taking one of the most inhibiting saccharides as 1.0. As hemagglutination assays are not easily quantitated, the results obtained remain an approximation. The assay described above allows the precise determination of the 50% inhibition concentration. In this way, small differences in inhibitory potency between sugars are readily established.

The assay was developed using asialotransferrin as the coated glycoprotein and RCA as the lectin. The results are comparable to those obtained with precipitation-inhibition or hemagglutination-inhibition assays (Goldstein et al., 1978). A major difference is the inability of fucose to inhibit binding in the assay, while it inhibits the turbidity resulting from the lectin-galactomannan reaction. A possible explanation might be found in the purity of the mono-

saccharides used: a slight contamination of a non-inhibiting sugar (i.e. fucose) with an inhibiting sugar (i.e. galactose) renders the material used inhibitory. Another explanation could be the presence of fucose-containing structures on the erythrocytes used to which the lectin binds in the absence of free fucose. The asialotransferrin used in our assay contains no fucose groups.

In principle our system is also suitable to characterise the binding of univalent lectins that are unable to induce hemagglutination. As hemagglutination is most often used to detect lectins, the existence of univalent lectins is still uncertain. The lectin-associated toxins that were once thought to be univalent may appear to have multiple binding sites (Housten et al., 1982).

In conclusion, our lectin-enzyme binding inhibition assay may be used to get a quick impression of the lectin-enzyme binding specificity of new lectins. Extending the number of inhibiting saccharides increases the correctness of the description.

4.3.1. *The determination of equivalency between lectin-biotin and streptavidin-peroxidase without using the lectin binding function*

The very high affinity of avidin or streptavidin for biotin and the simplicity of protein biotinylation have resulted in the widespread use of the avidin-biotin amplification system in histochemistry, cytochemistry and immunochemistry. The system employs in its basic form a complex of biotinylated binding protein (i.e. an antibody, a lectin, protein A, etc.), avidin or streptavidin, and biotinylated enzyme (i.e. peroxidase), or chemically conjugated avidin and enzyme. This complex may be prepared before use, and it is added in a single incubation step to the tissue, the cells or the immunoassay tubes in question.

The use of the avidin-biotin interaction in lectin binding studies has a few advantages. Labelling of lectins by radioactive iodine is circumvented. The introduction of the relatively large iodine molecules into the lectin protein induces changes in the binding characteristics (Shimoda et al., 1985). In comparison, the coupling of biotin molecules through a spacer arm to the lectin protein is a relatively inert modification: no changes in lectin binding affinity have been described.

Moreover, the biotinylation of the lectin and the use of avidin or streptavidin with four biotin binding sites allows the formation of large complexes. This may be advantageous in view of the relatively low association constant of lectin monomers, which may be overcome by creating multiple interactions between glycoprotein or cell membrane and lectin aggregates. It also provides an amplification which is a necessity when small amounts or subgroups of a glycoprotein or glycolipid are to be detected.

The use of streptavidin has a few advantages over avidin. Streptavidin has no carbohydrate groups to which lectins may bind. Moreover, its isoelectric point is 6.5 compared with 10.5 for avidin, leading to less nonspecific interactions

with negatively charged groups such as sialic acids. Both proteins possess four biotin binding sites (avidin groups).

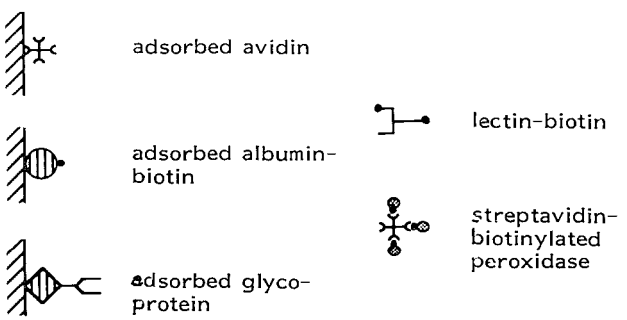
Complexes of streptavidin and enzyme, either linked chemically or by virtue of biotinylation of the enzyme, are commercially available, and biotinylated lectin has only to be added in equivalent amounts to prepare the desired complexes. However, this point of equivalency is not easily found.

In a sense, complexes of streptavidin-peroxidase (STAP) and lectin-biotin may be compared with antigen-antibody complexes where antigen excess, equivalence, and antibody excess are distinguished. Thus, in situations of STAP excess, the complexes formed will still have vacant avidin groups able to bind to biotin. In situations of lectin-biotin excess, the avidin groups will be saturated with lectin-biotin which will also be present in solution in free form. In situations of equivalence, all STAP avidin groups will be occupied by lectin-biotin, but no excess of either lectin-biotin or STAP will be present in solution. For lectin-enzyme binding assays this is the desired situation giving the largest complexes and giving the maximum color development.

An excess of free (biotinylated) lectin will effectively compete with lectin-STAP complexes for the available carbohydrate structures of the glycoprotein or glycolipid, leading to a loss of enzyme signal. An excess of free STAP on the other hand may increase the nonspecific binding or it may decrease the sensitivity due to a shortage of lectin-STAP complexes. Until now, no method has been described to provide a fast means of finding the point of equivalency where neither lectin nor STAP exists in excess.

Many lectins bind to only a few purified (and immobilised) glycoproteins, for instance from plasma. If no binding occurs the formation of optimal lectin-streptavidin-peroxidase complexes cannot be determined in a system based on the lectin binding function. Alternative and generally applicable methods must be sought to establish the optimal lectin-STAP complex composition for any lectin, independent of the lectin binding specificity.

It was reasoned that complexes formed in STAP excess will bind to a biotinylated solid phase, such as albumin-biotin coated to microtiter plates (figure 4-4).



Legend to figures 4-4, 4-5 and 4-6.

Figure 4-4.

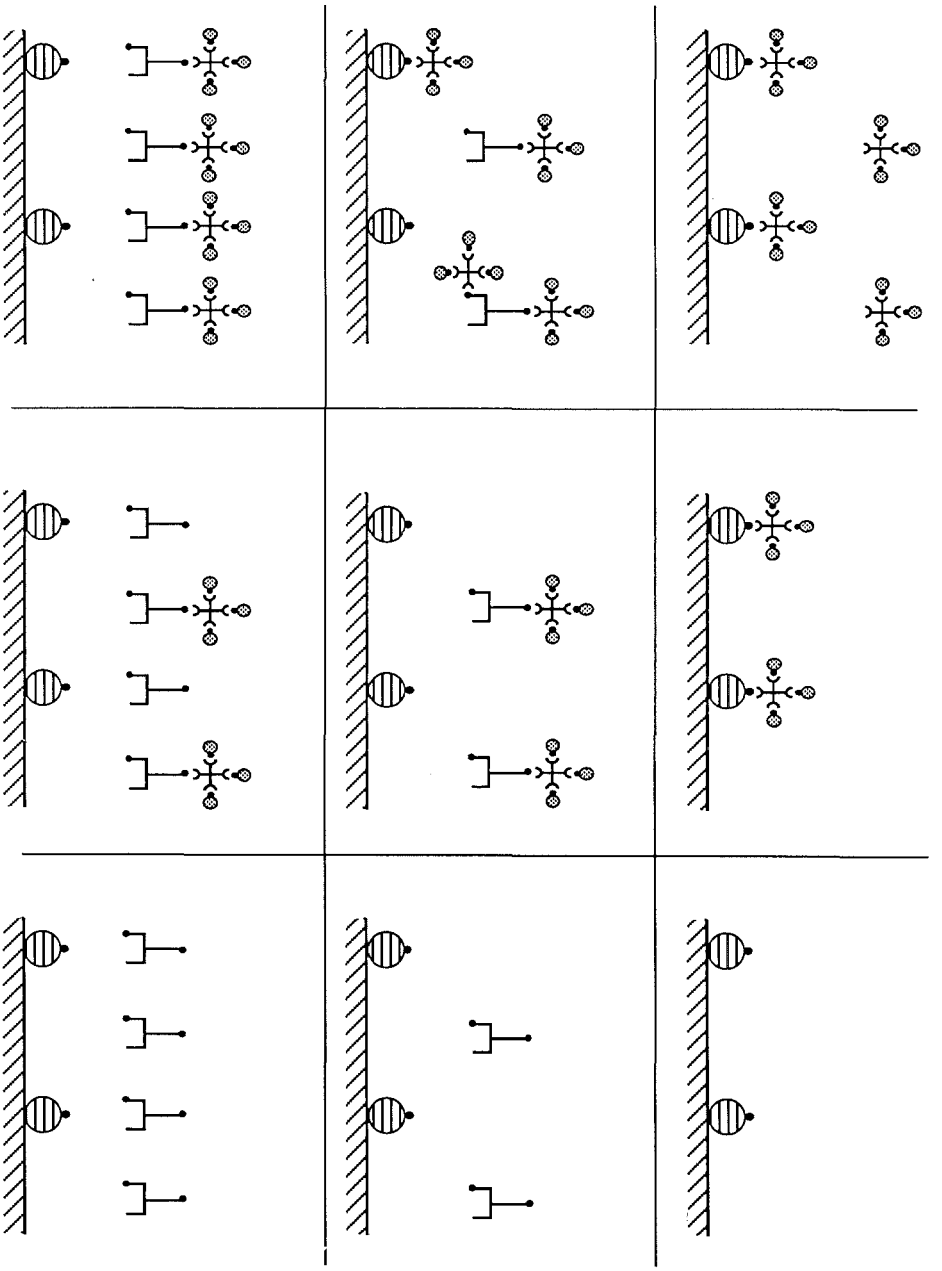
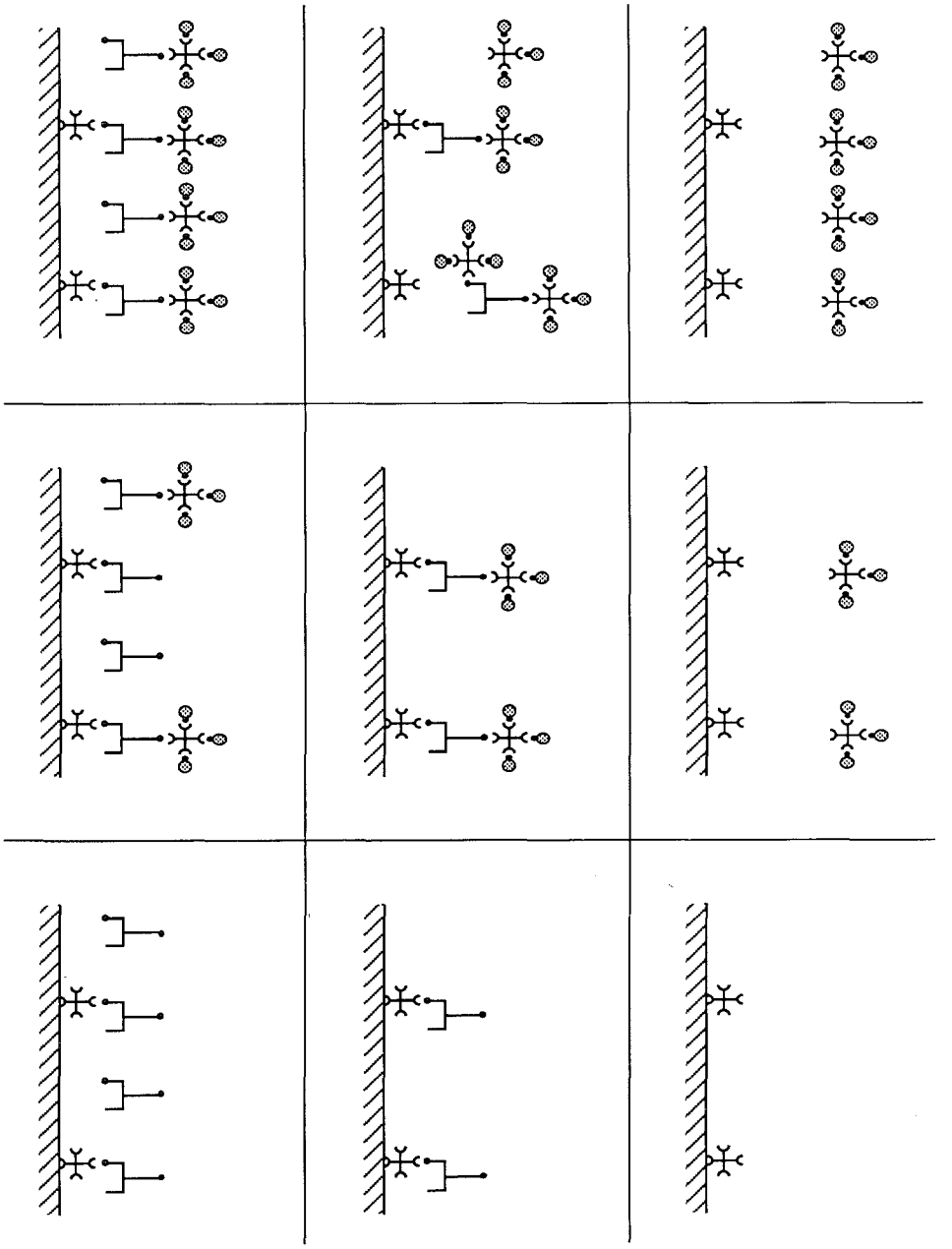


Figure 4-5.



At lectin-biotin excess on the other hand, not much STAP will be able to bind to albumin-biotin because all avidin groups on STAP are occupied by lectin-biotin. At equivalence, some binding of STAP complexes will occur due to the presence of a few STAP or lectin-STAP complexes possessing avidin groups that are left unoccupied. The optimal amounts of lectin-biotin and STAP may thus be approached in this way, for instance by searching for the half-maximal peroxidase enzyme activity bound to immobilised albumin-biotin.

By contrast, when using avidin coated plates quite different results are expected. In situations of STAP excess, as figure 4-5 shows, no binding will occur due to the absence of available biotin groups on the STAP complexes. In situations of lectin-biotin excess no binding of STAP will occur either because all immobilised avidin groups will be occupied with lectin-biotin. However, at equivalence some binding is to be expected due to the presence of available biotin groups on the lectin-biotin molecules bound to the STAP complexes, assuming more than one biotin group per lectin molecule.

We thought that it might be possible to determine the best STAP and lectin-biotin dilutions, for any biotinylated lectin, by using avidin coated microtiter plates. To investigate the feasability of this method the binding of preformed lectin-biotin-STAP complexes of different compositions was determined, both to avidin and to albumin-biotin coated microtiter plates, as well as to reference plates coated with a glycoprotein for which the lectin has affinity (figure 4-6). We thus used the combination of desialo-α_1-acid glycoprotein (desialo-AGP) coated plates and the lectin from Ricinus communis (RCA) which binds to desialo-AGP to determine if optimal binding in the desialo-AGP coated plates correlates with results obtained with avidin coated plates. If so, avidin (or streptavidin) coated microtiter plates may serve as a general tool to determine the best composition of complexes of STAP and biotinylated lectin (or other (binding) proteins such as antibody or protein A) without using the lectin binding function.

4.3.2. *Materials and methods*

4.3.2.1. Avidin coating of microtiter plates

Hen egg white avidin (Sigma Chemical Company, St. Louis, USA) was dissolved in PBS to a concentration of 5 μg/ml. 50 μl was transferred to each well of a microtiter plate (Flow laboratories, Irvine, Scotland). One row was left uncoated. After 1 hour at room temperature the plates were washed four times with PBS containing 0.05% Tween-20, tapped empty on paper tissue and allowed to dry. Plates were wrapped in aluminium foil and stored in -20°C until use.

4.3.2.2. Preparation of microtiter plates coated with perchloric acid soluble AGP

To human plasma an equal volume of cold 0.6 M perchloric acid was added.

Figure 4-6.

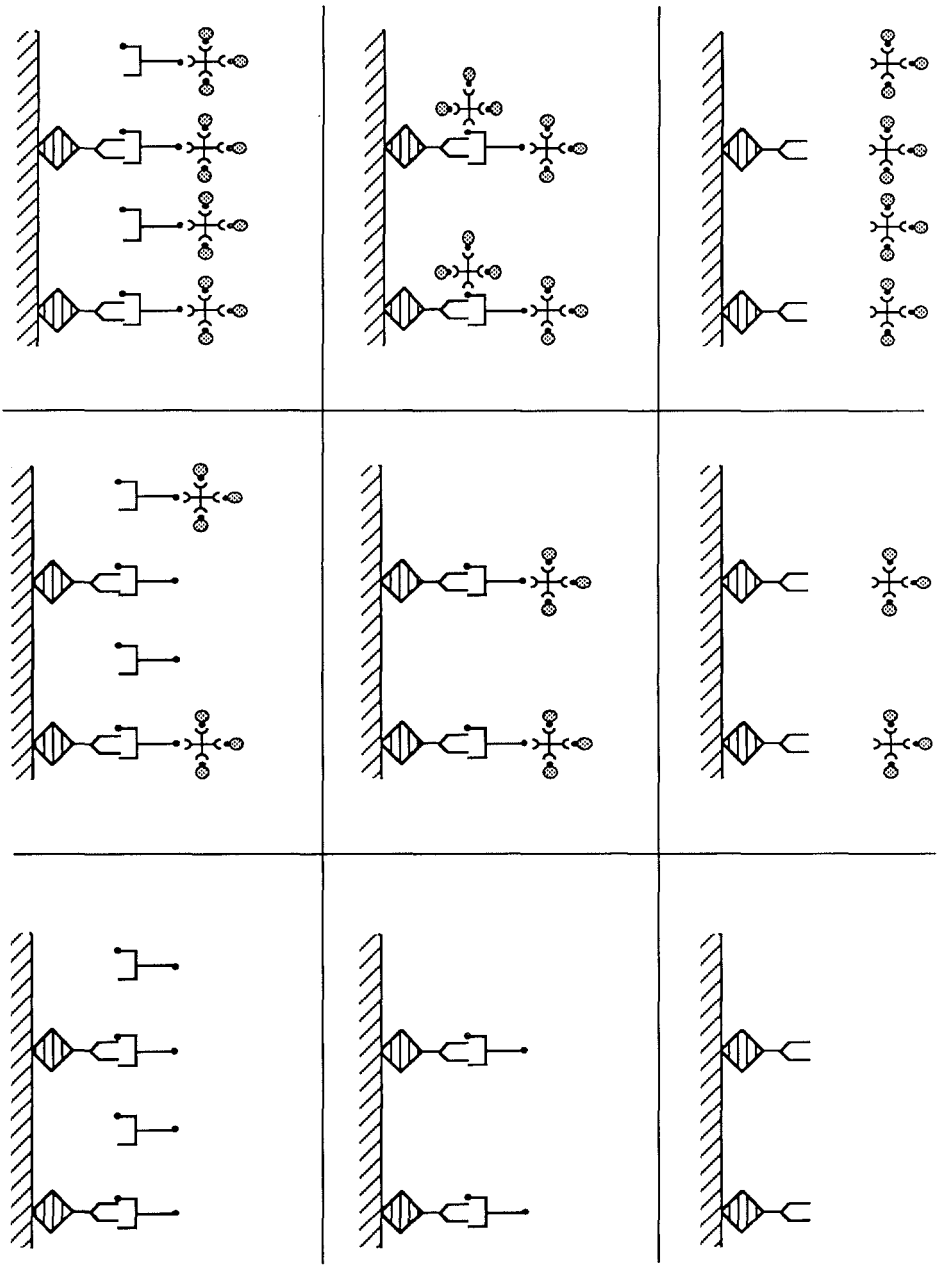


Figure 4-4, 4-5 and 4-6: Schematic representation of (RCA)-STAP binding to microtiter plate wells coated with albumin-biotin, avidin or glycoprotein, respectively, as a function of RCA-biotin dilutions (left to right) and STAP dilutions (top to bottom).

The precipitate was removed by centrifugation. The supernatant was dialysed against PBS. It consists mainly of AGP with diminished sialic acid content due to the acid treatment, together with traces of other proteins. The solution was diluted to 50 μg/ml. For coating the plates were treated as described above.

4.3.2.3. Preparation of albumin-biotin coated microtiter plates

Human albumin (KabiVitrum, Stockholm, Sweden) was biotinylated as described below for the lectins. The biotinylated albumin was diluted to a concentration of 5 μg/ml, and coating was proceeded as described above.

4.3.2.4. Biotinylation of lectin

The lectin from Ricinus communis was obtained from E.Y. Laboratories, San Mateo (Calif., USA). This and other lectins were dissolved in 0.1 M carbonate buffer pH 9.5 at a concentration of 10 mg/ml. 200 μl 0.1 M NHS-LC-Biotin (Pierce Chemical Company, Rockford, Illinois, USA) in 0.1 M carbonate buffer was added. After one hour at room temperature the mixture was applied on a 10 x 1.5 cm (length x diameter) gel filtration column containing polyacrylamide gel (Desalting Bio-Gel, Biorad, Richmond, USA) to remove the excess free biotin. The column separation procedure was standardized with a mixture of albumin (11 mg/ml) and tryptophan (1 mg/ml). The biotinylated lectin was stored in 200 μl aliquots in -20°C until use.

4.3.2.5. Preparation of lectin-STAP complexes

In an uncoated microtiter plate ten stepwise dilutions (1 + 1 in PBS) of the biotinylated lectin which were made separately in glass tubes were added in one direction ("horizontal") to six stepwise dilutions (1 + 1 in PBS) of STAP (Amersham International, Amersham, England), made separately, in the other direction ("vertical"). The complexes were allowed to form for 1 hour before transferring 50 μl of each complex to the corresponding well of each of the three microtiter plates coated with albumin-biotin, desialo-AGP and avidin, respectively. After incubation for 1 hour at 37°C the plates were washed four times with PBS-Tween and tapped empty on paper tissue. 160 μl of a freshly prepared chromogen solution of OPD and H_2O_2 in 30 mM citric acid, pH 5.5 was added.

Color development was allowed to take place for 20 minutes, after which the enzyme reaction was stopped by adding 40 μl 2.5 M H_2SO_4. Absorbance was read at 492 nm using a Titertek multiscan plate reader.

4.3.3. *Results*

The color development due to the bound peroxidase activity in the albumin-biotin coated microtiter plate is shown in figure 4-7. At high concentrations of biotinylated lectin no binding of STAP occurs. The extinctions are presented graphically in figure 4-8.
Figures 4-9 and 4-10 show the enzyme activity in the microtiter plate coated with desialo-AGP. Both lectin-biotin excess and STAP excess lead to less peroxidase binding.

The binding of lectin-STAP complexes to an avidin coated microtiter plate is shown in figure 4-11. Color development is only found at a fixed dilution ratio between biotinylated lectin and STAP. The extinctions are shown graphically in figure 4-12.

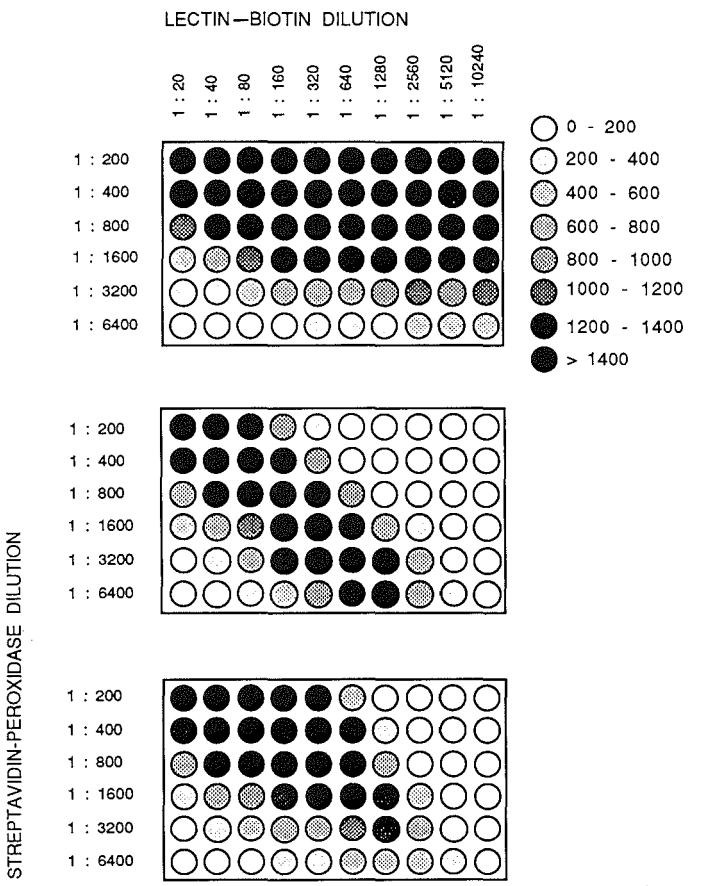
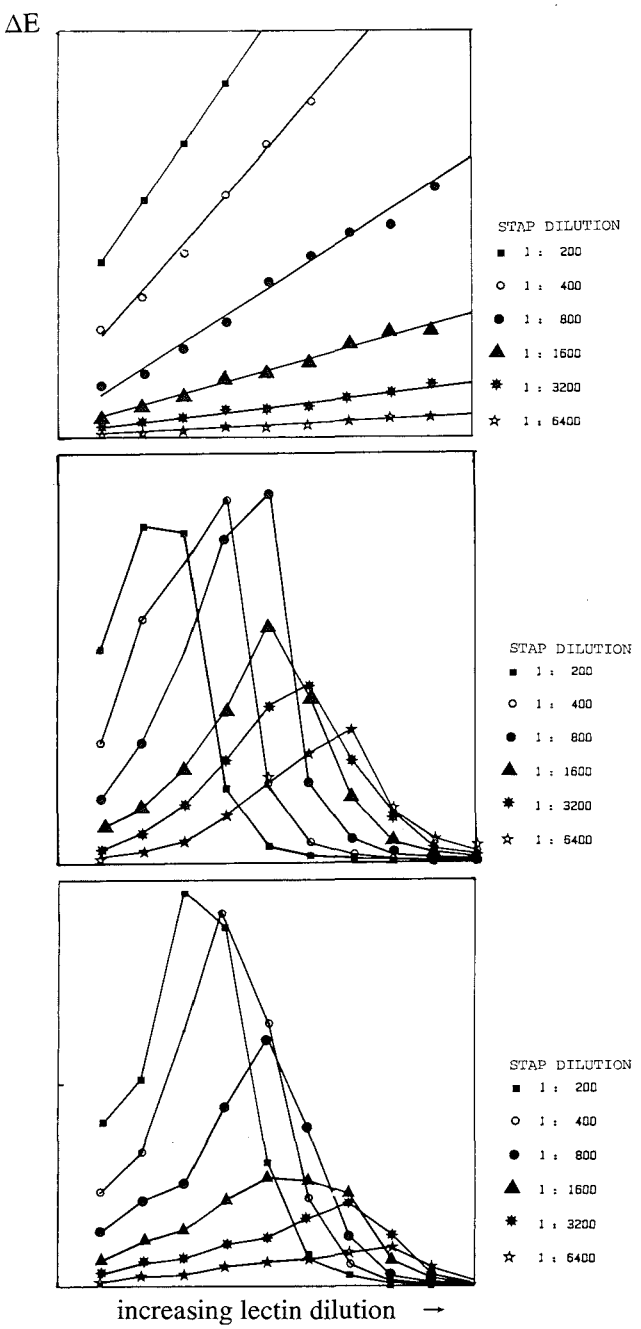


Figure 4-7 (top), 4-9 and 4-11 (bottom). Color development as a function of STAP and RCA-biotin dilutions in microtiter plates coated with albumin-biotin, avidin and desialo AGP, respectively.



Figures 4-8 (top), 4-10 and 4-12. Graphic representation of the binding of RCA-STAP complexes to microtiter plate wells coated with albumin-biotin (top), avidin or desialo AGP (bottom) as a function of STAP and RCA-biotin dilutions.

4.3.4. *Discussion and conclusions*

In the microtiter plate coated with desialo-AGP the RCA-lectin is the only constituent that binds, as neither streptavidin nor peroxidase have affinity for AGP. As the streptavidin-peroxidase complex is the only constituent with peroxidase activity, the binding of peroxidase to the plate can only be the result of complex formation between lectin and STAP, followed by binding of the complex through the lectin function. The results obtained with the glycoprotein coated plate indicate that the lectin dilution is not very critical. This is probably due to the stronger binding interaction of lectin molecules complexed by streptavidin, allowing multiple interactions with the coated glycoprotein, in comparison with the free lectin molecules that exist in solution in the case of lectin excess.

In the avidin coated plate the situation is slightly different. Here, too, free STAP will not be bound. Free lectin may bind to the avidin glycans but this does not lead to bound enzyme activity. Color development only takes place after binding of the lectin-STAP complex, either through the lectin which may bind to the carbohydrate chains of avidin, or through the excess biotin groups located on the lectin molecules in the lectin-STAP complex leading to recognition by the coated avidin. In the avidin coated plate the dilutions of lectin and STAP are more critical. This is expected as due to the extremely high association constant of the avidin-biotin interaction the biotinylated free lectin molecules that exist in lectin excess will in this case quite effectively compete with lectin-STAP complexes for the available avidin binding sites. Thus, a small amount of lectin excess can be tolerated in the glycoprotein coated plate, although it will lead to less binding in the avidin coated plate.

In the albumin-biotin coated plate effective binding of STAP is observed, as expected. Biotinylated lectin occupies the available biotin binding sites on STAP leading to a gradual loss of STAP binding to the coated albumin-biotin at increasing lectin-biotin concentrations.

For a few lectins the picture may be more complicated than as described above. In these cases the lectin binds to the carbohydrate groups of the peroxidase molecules in STAP. This interaction may have two results. First, the lectin may increase the process of multimerisation of STAP complexes due to its multivalency (most lectins are di- or tetravalent). This process adds to the complex formation already mediated by the multiple biotin groups on the lectin to which STAP complexes may bind, but the interaction is much weaker: Ka = 10^4 vs 10^{15} M^{-1}. Second, the binding of the STAP complex to the glycan of the immobilised glycoprotein may also be mediated through interaction of the lectin with the carbohydrate groups of peroxidase. In such cases the biotinylation of the lectin may not be necessary at all.

If one wishes to avoid this situation one might think of using a nonglycosylated marker enzyme, such as lactate dehydrogenase or E. coli alkaline phosphatase. However, the indicated interactions are probably not at all a disadvantage. It should be noted that this system only works when the lectin molecules contain

two or more biotin groups. Commercially available lectins contain on the average 3-8 biotin molecules.

The optimal ratios of lectin and STAP are those leading to high peroxidase activity in the avidin coated plates, and leading to a low peroxidase activity in the albumin-biotin coated plates. Given the results from the AGP coated plates we prefer to use in our subsequent experiments a two-fold or four-fold excess of lectin-biotin. After establishment of the ratio of lectin solution and STAP solution, the concentration of the formed lectin-STAP complex is subsequently chosen in such a way that an extinction of 1.0 is reached in 20 minutes. At higher STAP dilutions, the ratio becomes less critical. For most lectins we used 1:500 diluted biotinylated lectin solution (of 0.25 mg /ml) and 1:1000 diluted STAP, which equals roughly 0.5 μg of lectin per ml of 1:1000 diluted STAP. Thus, per well 25 ng of biotinylated lectin is used. 1 mg of biotinylated lectin suffices for 400 microtiter plates.

We also compared avidin and streptavidin coating of microtiter plates (results not shown). Avidin coated plates yielded more color development, possibly due to a more efficient coating of avidin with its high pI compared with streptavidin. Avidin also has the advantage of being readily avialable. Thus, in practice the right dilutions of biotinylated lectin and of STAP are found on the "diagonal" line linking points of equal lectin /STAP ratio in the avidin coated plate as shown in figure 4-5, a line which corresponds with the decreased binding of STAP in the albumin-biotin coated plate as shown in figure 4-4. We have now successfully used this method with more than thirty lectins.

In conclusion, the use of microtiter plates precoated with avidin and albumin-biotin allows the fast determination of the equivalent amounts of biotinylated lectin and streptavidin-peroxidase as well as the optimal dilution of the complex formed, without using the lectin binding function. The method may also be used with other biotinylated binding proteins, such as antibodies or protein A. The method will be applicable in histochemistry, cytochemistry and immunochemistry.

4.4.1 *Determination of lectin specificity by investigating the lectin-enzyme binding to various neoglycoproteins absorbed in microtiter plate wells.*

Lectins are detected by their capacity to agglutinate red blood cells. In most cases the structures of the sugar groups on the erythrocyte membrane to which they bind are not known. Inhibition studies with simple sugars, such as described in chapter 4.2 may give an indication with respect to the binding specificity of the lectin. However, while some lectins bind specifically to certain terminal monosaccharides, most lectins show higher affinities to complex structures, often extending to three or more sugar sequences. It is also important to note that the oligosaccharide structure involved in the binding of a lectin may be quite different from the best monosaccharide inhibitor.

Given the large diversity of the erythrocyte glycolipids and glycoproteins, it is reasonable to assume that most lectins will bind to a number of such structures,

with each interaction having its own association constant. When adding a competing saccharide the weak interactions will be inhibited first. Hemagglutination inhibition studies are thus only leading to an approximation of the binding specificity of the lectins. The bound structures are known only in the case of blood-group specific lectins.

A second method to investigate the binding specificity is to use lectins immobilised on the solid phase of a chromatography column. Radioactively labelled carbohydrates or glycopeptides are chromatographed and their retention times compared (Narasimhan et al., 1985; Osawa et al., 1987).

The second method has the advantage of using well-defined saccharides interacting with the immobilised lectins. However, the use of radioactive isotopes is a disadvantage. Besides, the lectin-column chromatography experiments are very time consuming. They also need a relatively large amount of lectin to be immobilised. These large amounts are not always available, as is the case of human membrane lectins.

In principle, lectin-enzyme binding studies do not have the drawbacks described above. If well-defined gycoconjugates are immobilised, a fast and unequivocal determination of the lectin binding specificity is possible. The amounts of material involved would be in the microgram range.

In this chapter experiments are described in which a number of well-defined BSA-glycoconjugates are adsorbed to microtiter plates. A large number of lectin-STAP complexes were allowed to bind. Subsequently, the amount of bound peroxidase activity was quantitated.

4.4.2. *Materials and methods*

The lectins used were selected from table 4-1.

The following BSA-glycoconjugates were obtained from Janssen Pharmaceutica (Beerse, Belgium) (table 4-2).

The BSA-glycoconjugates were dissolved in PBS. After measurement of the optical density at 280 nm they were further diluted to a final concentration of 5 μg/ml, assuming for all BSA-glycoconjugates an extinction of 0.53 for a 1.0 mg/ml solution. One lane was coated with avidin (Sigma, St. Louis, USA) at a concentration of 5 μg/ml. One lane was coated with biotinylated albumin at a concentration of 5 μg/ml.

Coating was allowed to take place in flexible PVC microtiter plates (Flow laboratories, Irvine, Scotland) for 1 hour. Subsequently, plates were washed five times with PBS containing 0.05% Tween 20, followed by a short incubation with PBS-Tween containing 5 μg/ml human albumin (Biomerieux, Lyon, France) and 0.05% v/v Triton X-100 (Technicon, Tarrytown, USA). Plates were tapped empty on paper tissue and allowed to dry. They were stored in -20°C wrapped in aluminium foil. The lectin-STAP binding protocol used is given in chapter 4.3.

Table 4-1. Lectins available for use in lectin-enzyme binding studies.

Lectins	Abbrev.	Cat no	Footnote
- Limulus polyphemus	LPA	L-1501	(a)
- Lotus tetragonolobus	Lotus	L-601	(a)
- Bauhinia purpurea	BPA	L-2501	(a)
- Maclura pomifera	MPA	L-3901	(a)
- Euonymus europaeus	EEA	L-4201	(a)
- Datura stramonium	DSA	L-5701	(a)
- Ulex europaeus I	UEA-I	L-2201	(a)
- Ulex europaeus II	UEA-II	L-2202	(a)
- Vicia villosa	VVA	L-4601	(a)
- Erythrina cristagalli	ECA		(b)
- Erythrina corallodendrum	ECO		(b)
- Tridacna maxima	TMA		(c)
- Solanum tuberosum	STA		(c)
- Crotalaria juncea	CJA		(c)
- Pisum sativum	PSA	L5380	(d)
- Phaseolus vulgaris Leucoagglutinin	PHA.L	L8504	(d)
- Phaseolus vulgaris eryagglutinin	PHA.E	L8629	(d)
- Lens culinaris	LCA	L5880	(d)
- Arachis hypogaea	PNA	L0881	(d)
- Ricinus communis I	RCA	L8508	(d)
- Bandeiraea simplicifolia II	BSII	L7508	(d)
- Concanavalin A	Con A	17-0450-01	(e)
- Triticum vulgaris	WGA	17-0750-01	(e)
- Aleuria aurantia	AAL		(f)
- Limax Flavus	Limax		(g)
- Abrus precatorius	APA		(d)
- Artocarpus integrifolia	AIA (Jacalin)		(d)
- Anguila anguila	AAA		(d)
- Phylodendron californium	PCA		(h)
- Artocarpus communis	ACA		(i)

Sources:

a. Gift from E.Y. laboratories, San Mateo, California. USA.

b. Gift from Prof. N. Sharon, Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel.

c. Gift from Prof. G. Uhlenbruck, University of Köln, West-Germany.

d. Obtained from Sigma Chemical Company, St. Louis, USA.

e. Obtained from Pharmacia, Uppsala, Sweden.

f. Gift from Dr. H. Debray, Université de Lille, Lille, France.

g. Gift from Prof. J. Angeray, Université de Paris Sud, Paris, France.

h. Gift from Prof. H. Franz, Staatliches Institut für Immunpräparate und Nahrmedien.

i. Gift from T. Animashaun, MRC, London, England.

Table 4-2. Available BSA-glycoconjugates.

Cat. nr	Molar chain ratio	Structure
B-1000	30 - 40	CH_3-
B-1001	20 - 30	Glcβ-
B-1002	30 - 40	Galβ-
B-1003	30 - 40	Manα-
B-1004	20 - 30	Galβ1-4Glcβ-
B-1005	20 - 30	Galβ1-4GlcNacβ-
B-1006	30 - 40	Galα1-4Galβ-
B-1007	10 - 20	Glcα1-6Glcα1-4Glcα1-4Glcβ-
B-1008	10 - 30	GlcNAcα1-4{Fucα1-6}GlcNAcβ-
B-1009	15 - 30	Galα1-4Galβ1-4Glcβ-
B-1010	20 - 40	Galα1-4Galβ1-4GlcNAcβ-
B-1011	10 - 20	Galβ1-3GalNAcβ-
B-1012	25 - 35	Galβ1-3GlcNAcβ-
B-1013	30 - 40	GlcNAcβ-
B-1014	20 - 30	GalNAcβ-

4.4.3. *Results*

The peroxidase enzyme activity found in the glycoconjugate-coated wells is compared with the activity found in the avidin-coated wells. This latter activity is taken as 100%, and expressed in table 4-3 as ++. Higher activities are +++. Between 20% and 100% of the avidin signal is given +, and between 10% and 20% is given ±. Below 10% is considered negative: —.

4.4.4. *Discussion and conclusions*

The method described in this chapter allows the fast and detailed determination of the binding specificity of the lectin used. The lectin-STAP complex is simply added to all wells, and within a few hours the results are available. The plates coated with the BSA-glycoconjugates are stored in the freezer. We observed no loss in binding of any of the lectins, indicating stability of the coated and dried plates. This allows the coating at one time of a large number of plates for later use, thus contributing to the efficiency of the method described. For RCA, representation of the results as follows leads to a few new findings.

From these results it may be concluded that RCA may bind slightly to innerchain -4Galβ1-4Glc(NAc)β-structures. The addition of terminal α1-4 galactose has a much larger influence on the binding to the lactosamine group than to the binding to the lactose group. It is also apparent that the introduction of a glucose group between the terminal β-galactose and the carrier protein slightly inhibits binding of RCA, but that a subsequent addition of an N-Acetyl group to the glucose increases the binding to higher than original values.

Table 4-4. RCA-STAP binding to some BSA-glycoconjugates.

Sugar	Molar BSA /glycan ratio
Galβ - +++	30 - 40
Galβ1 - 4Glcβ - ++	20 - 30
Galβ1 - 4GlcNAcβ - ++++	20 - 30
Galα1 - 4Galβ1 - 4Glcβ - +	15 - 30
Galα1 - 4Galβ1 - 4GlcNAcβ - +	20 - 40
Galα1 - 4Ga1β - 0	20 - 40

Not all BSA-glycoconjugates carry exactly the same number of saccharide chains per BSA molecule. It is not expected, though, that this number has a significant influence on the results, as the BSA-glycoconjugate can only accomodate a few lectin-STAP complexes at the most. The molar glycan ratio will therefore be effectively in excess in all cases.

The method is validated by the fact that lectins with known indentical specificities show exactly the same binding pattern in BSA-glycoconjugate coated microtiter plates. For instance, the lectin called Jacalin from the jackfruit (Artocarpus integrifolia) from Venezuelan sources shows the same binding pattern as the lectin from the breadnut (Artocarpus communis) from Nigerian sources, although no doubt the plant species, the isolation procedures and purity are different. The lectins LCA and PSA with slightly different binding specificities (Kornfeld et al., 1981) both bind to none of the BSA-glycoconjugates. ECA and ECO, isolated from different Erythrina species, bind only to Galβ1-4GlcNAcβ structures. For the other lectins, the known binding specificities (Goldstein et al., 1978; Liener et al., 1986) are confirmed by our assays. However, in a number of instances new or extended affinities are found. For example, the binding of Jacalin and ACA, known to be Gal/GalNAc specific, to Mannose and $(Glc)_4$ as well as to GlcNAcβ1-4 (Fuc α1-6) GlcNAcβ is a new finding. It appears that our assay is more sensitive than other techniques leading to the detection of weak interactions not found by hemagglutination or lectin-column chromatographic techniques.

A similar approach has been used for studying the lectin binding specificity to glycosphingolipids (Molin et al., 1986). The lipids were coated onto microtiter

Table 4-3. Binding of 30 lectin-STAP complexes to 14 immobilized BSA-neoglycoconjugates.

	DSA	RCA	WGA	ECA	ECO	LCA	PNA	TMA	AAA	Limax flavus	APA	WA	Lotus	ACA	BSA
B-1000 = CH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-1001 = Glcβ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-1002 = Galβ	-	+++	-	-	-	-	-	-	-	-	+++	-	-	-	-
B-1003 = Manα	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
B-1004 = Galβ 1-4Glcβ	-	++	-	-	-	-	-	-	-	-	+++	-	-	-	-
B-1005 = Galβ 1-4GlcNacβ	+	+++	+++	+++	+	-	-	-	-	-	+++	-	-	-	-
B-1006 = Galα 1-4Galβ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-1007 = Glcα 1-6Glcα 1-4Glcα 1-4Glcβ	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-
B-1008 = GlcNacβ 1-4(Fucα 1-6)GlcNacβ	-	-	+++	-	-	-	-	-	+	-	-	-	-	±	+
B-1009 = Galα 1-4Galβ 1-4Glcβ	-	+	±	-	-	-	-	-	-	-	+++	-	-	-	-
B-1010 = Galα 1-4Galβ 1-4GlcNacβ	±	+	+++	-	-	-	-	-	-	-	++	-	-	-	-
B-1011 = Galβ 1-3GalNacβ	-	++	±	-	-	-	++	-	-	-	+++	±	-	++	-
B-1012 = Galβ 1-3GlcNacβ	-	+	-	-	-	-	-	-	-	-	+++	-	-	NT	NT
B-1013 = GlcNacβ	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	+
B-1018 = GalNacβ	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-

	LPA	PSA	PHA-L	STA	BPA	MPA	EEA	PHA-E	Con-A	CIA	Ulex-1	Ulex-2	Jacalin	AAL	PCA
B-1000 = CH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-1001 = Glcβ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-1002 = Galβ	-	-	-	-	±	-	-	-	-	-	-	-	-	-	++
B-1003 = Manα	-	-	-	-	-	-	-	-	+++	-	-	-	+	-	-
B-1004 = Galβ 1-4Glcβ	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
B-1005 = Galβ 1-4GlcNacβ	-	-	-	-	±	-	-	-	-	++	-	-	-	-	+++
B-1006 = Galα 1-4Galβ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-1007 = Glcα 1-6Glcα 1-4Glcα 1-4Glcβ	-	-	-	-	-	-	-	-	++	-	-	-	±	-	-
B-1008 = GlcNacβ 1-4(Fucα 1-6)GlcNacβ	-	-	-	-	-	-	-	-	-	-	-	-	±	+++	-
B-1009 = Galα 1-4Galβ 1-4Glcβ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-1010 = Galα 1-4Galβ 1-4GlcNacβ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-1011 = Galβ 1-3GalNacβ	-	-	-	-	+++	-	-	-	-	-	-	-	++	-	-
B-1012 = Galβ 1-3GlcNacβ	-	-	-	-	++	-	-	-	-	NT	NT	NT	NT	NT	NT
B-1013 = GlcNacβ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-1018 = GalNacβ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

plates by evaporating the solvent from a cholesterol-lecithin-lipid solution. Biotinylated lectins were allowed to bind. After washing, avidin-peroxidase conjugate was added and the amount of bound peroxidase subsequently quantitated.

The method described in this chapter would not have the problems associated with coating microtiter plates with glycosphingolipids. Such a coating has to be made by evaporating the solvent (i.e. methanol) from a certain critical mixture of glycolipid, cholesterol and lecithin. The BSA-glycoconjugates that we used show excellent passive adsorption from solution onto the microtiter plates. When BSA-lipid conjugates become available they could be used to investigate lectin binding to lipids.

A second difference between the two methods is the addition in our assay system of preformed lectin-streptavidin-peroxidase complexes, in contrast to the addition of biotinylated free lectin. In view of the possible resolubilisation of bound lectin molecules during wash cycles (Cerven et al., 1981), we preferred the complexing of lectin molecules through streptavidin before use. In our method no loss during wash cycles was observed. This was not tested in the other method.

A reversed technique has been developed recently, in which the lectins RCA, WGA and Con A are immobilized to Sepharose and BSA-glycoconjugates are passed over the lectin column. Maltose BSA, lactose-BSA and $(GlcNAc)_2$-BSA were bound to immobilized Con A, RCA and WGA-Sepharose, respectively (Ito, 1985). In principle, the results obtained with lectin column chromatography of BSA-glycoconjugates should be comparable with our lectin-enzyme binding assays in BSA-glycoprotein-coated plates.

At present only a relatively small number of BSA-glycoconjugates are available for testing. This still limits the application of the method to those lectins that bind to one or more of the available glycoconjugates. However, in the near future much more glycoconjugates will certainly become commercially available.

A second limitation that exists at present is found in the structures of the glycoconjugates. These structures are synthesized in the test tube, and not all of them occur linked to plasma glycoproteins. Although they are useful as indicators of the binding specificity of the lectins, they may or may not give information with respect to the binding of lectins to naturally occuring glycovariants of N-linked and O-linked glycans. It is expected that this limitation will be overcome in the near future in two ways. Firstly, an abundantly available glycoprotein with one or two simple glycans (i.e. transferrin) may be used to prepare a set of glycovariants. The neccessary enzymes (i.e. neuraminidase, β-galactosidase, β-N-acetylglucosaminidase, α-fucosidase, etc.) are at present available or becoming available. The separation and purification of the glycovariants may be accomplished by chromatofocusing or anion-exchange chromatography if sialic acids are involved (chapter 3), and by lectin affinity chromatography.

Secondly, the enzymatic removal of complete glycans from glycoproteins by enzymes such as N-glycanase, O-glycanase and Endo F allows the production

on a preparative scale of free glycans, which may subsequently be coupled by chemical means to inert carriers such as BSA.

In conclusion, the binding of lectin-enzyme conjugates to microtiter plates coated with BSA-glycoconjugates enables the specificity of lectins towards several carbohydrate units to be investigated rapidly.

4.5.1. *The investigation of lectin-enzyme binding to purified human plasma glycoproteins adsorbed in microtiter plate wells*

In order to achieve a high sensitivity of lectin-enzyme binding assays in a clinical setting, the lectin used should not bind to the glycoprotein in plasma from healthy subjects. It should bind, however, to the (minor) fractions of the glycoprotein that are abnormally glycosylated during disease. Thus, a suitable lectin should meet two conditions. Firstly, there should be little binding to the normally glycosylated protein. This condition is explored in the experiments described here. Secondly, the lectin should bind to the glycoprotein in certain disease states. This condition is explored in chapter 5 where a number of lectins are tested for their ability to discriminate between a glycoprotein from healthy subjects and from a number of patients.

Various ways exist to investigate the binding of a lectin to glycoproteins present in normal plasma. One might think of affinity chromatography of whole plasma over immobilised lectin columns (chapter 2), or crossed affino-immunoelectrophoresis with a lectin in the first dimension. These techniques are very laborious and large amounts of lectin are needed. It was therefore decided to explore the lectin-enzyme binding in microtiter plates coated with the major human plasma glycoproteins in purified state. This technique needs very small amounts of the purifed glycoproteins because complete adsorption is achieved with approximately one microgram per well. The technique also uses only extremely small amounts of the lectin: nanograms per well. The ease and speed of the assay were also considered to be superior over other techniques, allowing the screening of a large number of lectins.

Microtiter plates were coated with eighteen plasma glycoproteins purified from normal plasma. The binding of 30 biotinylated lectin-STAP complexes was quantitated.

4.5.2. *Materials and Methods*

The lectins used were selected from table 4-1. The human plasma proteins used were those from table 4-5.

Table 4-5. Purified human plasma proteins used in lectin-enzyme binding studies.

Protein	Cat. no.	Footnote
Lactoferrin	K.Nr.190875	(a)
Thyroxin-binding Globulin	K.Nr.271184	(a)
Hemopexin	K.Nr.170382	(a)
Gc-Globulin	K.Nr.241277	(a)
8Sα_3-Glycoprotein	K.Nr.150482	(a)
α_2-HS-Glycoprotein	K.Nr.091285	(a)
Acid-α_1-Glycoprotein	K.Nr.080378	(a)
$\alpha_1\beta$-Glycoprotein	K.Nr.280180	(a)
Haptoglobin	K.Nr.280379	(a)
β_2-Glycoprotein	K.Nr.261182	(a)
Transferrin	K.Nr.040223	(a)
α_1-Antitrypsin	K.Nr.090379	(a)
Ceruloplasmin	K.Nr.291082	(a)
Immunoglobulin A	No. I-1010	(b)
Immunoglobulin G	No. I-4506	(b)
α_2-Macroglobulin	No. M-4514	(b)
Immunoglobulin M	No. 401107	(c)
Albumin	88377	(d)

Sources:
a. Gift from Behringwerke (Marburg, West-Germany).
b. Obtained from Sigma Chemical Company (St. Louis, USA).
c. Obtained from Calbiochem (Behring Diagnostics, La Jolla, USA)
d. Obtained from KabiVitrum (Stockholm, Sweden).

Coating of human plasma (glyco)-proteins to microtiter plates

All plasma proteins were dissolved in PBS to a final concentration of 5 μg / ml. 50 μl was transferred in duplicate to the microtiter plates obtained from Flow Laboratories.

After one hour the plates were washed four times with PBS containing 0,05% Tween-20, tapped empty on paper tissue and allowed to dry. Plates were wrapped in aluminium foil and stored in -20°C until use.

Biotinylation of lectins

Lectins were biotinylated as described under 4.3.2.4.

Determination of the optimal concentrations of biotinylated Lectin and Streptavidin-Peroxidase-Complex

This procedure is described under 4.3.2.5.

Lectin enzyme binding assay

For each lectin, 50 μl of the lectin-streptavidin-peroxidase (Lectin-STAP) complex was prepared as described above, and added in the microtiter plates precoated with the eighteen plasma proteins. Besides, one row was left uncoated (blank value). One row was coated with biotinylated albumin and one row was coated with avidin to check the composition and concentration of the lectin-STAP complex.

After 120 minutes incubation at 37°C, the plates were washed four times with PBS-Tween, and tapped dry on paper tissue. 160 μl of peroxidase reagent (OPD-H_2O_2) was added, and color development was allowed to take place at room temperature in the dark for 20 minutes. The reaction was stopped with 40 μl of 2.5 M H_2SO_4, and the absorbance read at 492 nm.

4.5.3. *Results*

The absorbance due to the peroxidase enzyme activity in the avidin coated lane is taken as 100%. Readings between 80% and 100% are expressed in table 4-6 as +++, higher values as ++++. Values between 50% and 80% are ++, between 80% and 20% are + and between 20% and 10% are ±.

4.5.4. *Discussion and conclusion*

The binding of lectin-STAP complexes to passively adsorbed glycoproteins is influenced by a number of variables. First, the density of the various coated glycoproteins is probably not the same because some glycoproteins coat "better" than others. Second, the glycan density on the molecule may be involved, as well as the spatial arrangement of these branches. Third, the presence of structures for which the lectin has affinity is a prerequisite for binding. Together, these three variables determine the amount of lectin bound to the microtiter plate well.

From the glycoproteins tested, IgM appears to bind to most lectins. This is probably due to a number of factors. First, in contrast to all other plasma glycoproteins, the immunoglobulins are very heterogenous with respect to their aminoacid sequence, and in consequence their glycosylation at the Fab-arms is extremely diverse. Many immunoglobulin molecules do not have any Fab-arm-linked glycans, while others have one or more of these glycans. On the average 0.8 glycan is present on the Fab-arm (Rademacher et al., 1984). Fc-glycosylation is heterogenous with respect to the glycan structures only. The number of Fc-linked glycans is fixed per immunoglobulin class.

Second, due to the pentameric structure of IgM, the number of glycans bound to one molecule is relatively large. This fact may facilitate the binding of lectins which have restricted spatial arrangements by allowing a large number of possible interactions. The same is probably true for α_2-macroglobulin, which has an

Table 4-6. Binding of 30 lectin-STAP complexes to 17 immobilized human plasma glycoproteins and albumin.

	ECA	ECO	LCA	PNA	IMA	CJA	VVA	Ulex1	Ulex2	Jacalin	ACA	Lotus	EEA	DSA	BSA
Lactoferrin	–	–	+++	–	±	+	–	++	+	±	–	–	–	–	+
Thyroxinb.Globulin	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Hemopexin	–	–	–	–	–	–	–	–	–	++++	++++	–	–	+	–
Gc-globulin	–	–	–	–	–	–	–	–	–	+	±	–	–	+	–
8Sα_3-Glycoprotein	–	–	+	–	–	–	–	–	–	++	+	–	–	++	–
α_2-HS-Glycoprotein	–	–	–	–	–	–	–	–	–	++++	++++	–	–	–	–
Acid-α_1-Glycoprotein	–	–	–	–	–	–	–	±	–	+++	++	–	–	+	–
α_1-β-Glycoprotein	–	–	–	–	–	–	–	±	–	+	–	–	–	–	–
Haptoglobin	–	–	–	–	–	–	–	–	–	–	–	–	–	++++	–
β_2-Glycoprotein	–	–	+	–	–	–	–	±	–	–	–	–	–	+	–
Transferrin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
α_1-antitrypsin	–	–	–	–	–	–	–	–	–	+	+	–	–	±	–
IgA	–	–	++	+	–	+	–	–	–	++++	++++	±	+	–	++
α_2-Macroglobulin	–	–	+	–	–	–	–	–	–	++	+	–	–	–	±
IgM	++	–	++	±	±	+	–	–	–	+	±	++	–	±	+++
Ceruloplasmin	–	–	+	±	±	±	–	–	–	–	–	–	±	±	–
Albumin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IgG	–	–	++	–	±	–	–	–	–	±	±	±	+	–	++

	LFA	PCA	AAL	PHA.L	RCA	WGA	PSA	STA	LPA	BPA	MPA	PHA-E	Con A	APA	AAA
Lactoferrin	−	+++	+++	−	+++	−	++	±	±	+	−	+	+	+++	++
Thyroxinb.Globulin	+	+	−	−	+++	+	−	−	±	±	−	−	+	+++	−
Hemopexin	−	+	−	−	+++	++	−	−	±	−	−	−	±	+++	−
Gc-globulin	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
8S α_3-Glycoprotein	++	+++	+	−	+++	++	+	−	±	−	−	−	++	+++	−
α_2-HS-Glycoprotein	−	+	−	−	++	+++	−	−	−	−	−	−	±	++	−
Acid-α_1-Glycoprotein	−	±	±	−	+	++	−	−	−	−	−	−	−	++	−
α_1-β-Glycoprotein	−	+	−	−	+++	−	−	−	−	−	−	−	±	+++	−
Haptoglobin	++	+++	−	+++	+++	−	±	−	−	−	−	−	++	+++	−
β_2-Glycoprotein	−	++	±	−	+++	+	+	−	−	−	−	−	+	+++	−
Transferrin	−	±	−	−	++	−	−	−	−	−	−	−	±	++	−
α_1-antitrypsin	−	+	−	−	++	±	−	−	−	−	−	−	±	+++	−
IgA	−	++	+++	−	+++	+++	++	+	±	+++	+++	++	+++	+++	−
α_2-Macroglobulin	+	+++	+	−	+++	++	+	±	±	−	±	+	−	+++	−
IgM	+	+++	++	±	+++	++	+++	+++	++	++	±	+++	+++	+++	+
Ceruloplasmin	+	−	−	−	++	±	−	±	−	−	−	−	±	++	−
Albumin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
IgG	−	++	++	−	+++	±	++	++	+	−	−	+	+	+++	−

estimated 23 glycans per molecule. If the intermolecular distances are large in comparison to the intramolecular distances between glycans, this aspect of glycan density is of importance. Third, IgM probably binds well to polystyrene surfaces due to its average pI and molecular weight. Some lectins bind even better to IgA than to IgM, i.e. PNA and MPA. No doubt this is caused by the presence of α1-3 linked galactose in O-linked glycans that occur in IgA_1. The excellent binding of BPA is also to be noted. The binding characteristics of these three lectins are similar, although not the same (Wu, 1984).

IgG shows slight binding to a large number of lectins. Here the glycan diversity mentioned above for IgM has the same effect.

α_2-macroglobulin also binds slightly to a large number of lectins. The described effect of molecular size and glycan density on lectin binding is of importance here. Absence of binding of α_2-macroglobulin to PNA and BPA is noteworthy.

Haptoglobin also shows a peculiar reaction pattern. Of the galactose-specific lectins, it shows very strong binding to RCA. This interaction is confirmed by the unique affinity of DSA for haptoglobin. DSA binds only to terminal galactose groups (Yamashita et al., 1987). On the other hand, ECA did not bind to haptoglobin, although it is also galactose-specific. A number of other lectins appear to bind to fractions of haptoglobin, or bind with lower affinities.

Two proteins did not show any binding to the lectins tested. One is albumin which is not a glycoprotein, thus no lectin binding is expected. It merely served as a control to detect any nonspecific binding of the lectin-streptavidin-peroxidase complex to the protein-coated wells. Gc-globulin (vitamin D binding protein or group-specific component) is bound weakly by Jacalin, ACA, DSA and APA. This protein, however, is a glycoprotein. It probably contains one non-sialylated N-linked glycan (Uhlenbruck et al., 1979). Apparently, this is not enough to bind most of the lectins. Its coating efficiency to polystyrene microtiterplates is unknown.

The broad specificity of RCA towards plasma glycoproteins, as described earlier by Uhlenbruck (1978) for plasma asialoglycoproteins, is confirmed by our results. Part of this reactivity may be explained by the low affinity of RCA for innerchain Galβ1-4-structures as in sialylated N-linked glycans (Baenziger et al., 1979, Narasimhan et al., 1986; see also chapter 4.4). In contrast to α2-6-linked, the α2-3-linked sialic acids allow some interactions with the penultimate galactoses, both with RCA I as with L-PHA and E-PHA lectins (Green et al., 1987a, b). WGA also seems to bind (fractions of) most glycoproteins; α_2-HS glycoprotein and IgA binding best. Monoclonal human IgG_3 interacts with RCA, in contrast to IgG_1, IgG_2 and IgG_4 (Saltvedt et al., 1975). α_2-macroglobulin, haptoglobin, IgM and IgA are retarded by immobilized RCA (Harboe et al., 1975).

The similar binding specificity of LCA and PSA (Kornfeld et al., 1981) is confirmed in this study. The binding to lactoferrin suggests the occurrence of fucose, probably a general finding in milk glycoproteins. The lectins Jacalin and ACA from two Artocarpus species (jackfruit and breadnut) also show a strong resemblance in binding.

Con A like WGA binds to (fractions of) most plasma glycoproteins, although its pattern is clearly different from WGA. Con A did not bind to α_1-acid glycoprotein, a finding contrasting with results obtained by crossed affino-immunoelectrophoresis (Hanson et al., 1984). The reason for this discrepancy is unknown. As WGA binds to AGP a low coating efficiency of AGP due to its extremely low pI of AGP (2.8) cannot be the reason. Con A is said to bind only to biantennary glycans without intersecting GlcNAc. However, if the sialic acids are missing, an intersected biantennary glycan is bound (Baenziger, 1985).

Of the lectins used, PNA was the most selective, binding only to IgA and traces of IgM. The limited binding of DSA lectin to all glycoproteins except haptoglobin suggests that during the purification of the glycoproteins from human plasma few sialic acids are lost. In contrast to RCA, DSA appears to have no affinity at all for inner chain galactose (Yamashita et al., 1987), just as the Erythrina lectins (Debray et al., 1986). Using lectin column chromatography it was shown that most haptoglobin and ceruloplasmin from normal serum bound to Con A (Mallet et al., 1987). 69% of α_1-antitrypsin bound, and 2% of AGP. However 72% of AGP and 27% of AAT were retarded, indicating a weak interaction with Con A. In the lectin-enzyme assay in its present form no distinction can be made between firm and weak binding. This problem may be solved by adding increasing amounts of the competing sugar to the assay. A loss of signal at low inhibitor concentration indicates a weak interaction, and a loss of signal at high inhibitor concentration indicates a strong interaction. These experiments remain to be done.

Baumstark (1983) also performed Con A column chromatography. α_2-macroglobulin and ceruloplasmin appeared to bind most strongly, that is, were eluted by the highest mannose concentration. In our assay IgA and IgM gave the highest binding, followed by α_2-macroglobulin, haptoglobin and $8S\alpha_3$-glycoprotein. Ceruloplasmin showed a weak binding in our assay. The reason for these discrepancies is at present unknown. The binding of plasma glycoproteins to the immobilized lectins PHA-E4 and PHA-L4 from the red kidney bean was recently investigated (Glad et al., 1984). Some haptoglobin bound to PHA-L4, while in our assay some IgM was bound. PHA-E4 bound best to IgM and haptoglobin, as well as α_2-macoglobulin, IgA and IgG, while in our assay IgM and IgA bound best, followed by α_2-macroglobulin and IgG.

In earlier studies no separation was made of the two PHA lectins which have quite different binding specificities. Using a column of immobilized PHA (containing unknown amounts of E4 and L4) the following plasma glycoproteins were found to bind (Spengler, 1981):

- IgM +++
- α_2-macroglobulin ++
- apo-B ++
- AGP +
- IgA +

In our assay the order would be:

- IgM +++
- IgA ++
- α_2-macroglobulin +
- apo B ?
- IgG +
- AGP —

This lectin-enzyme binding system appears to be more sensitive than precipitation assays. For instance, no precipitation was seen of transferrin with Con A (Young et al., 1974), although slight binding of Con A to adsorbed transferrin was detected in our assay. This binding is confirmed by lectin-column chromatography (Baumstark, 1983). On the other hand, the precipitation of transferrin and LCA found by the authors could not be reproduced by us: no binding of LCA-enzyme complex was detected in our system. This is to be expected given that the presence of core α1-6-linked fucose is a prerequisite for binding (Kornfeld et al., 1981) and the absence of fucose in human serotransferrin. Another source of discrepancies is the fact that binding does not necessarily lead to precipitation (Felsted et al., 1976; Bhattacharyya et al., 1988a,b), i.e. the absence of a precipitation reaction (arc formation in gels or turbidity formation in solutions) does not mean the lectin does not bind to the glycoprotein.

A good correlation is obtained between the results described above and those obtained with lectin-column chromatography as described in chapter 3. The selective binding of MPA to IgA is confirmed, but the lectin-enzyme binding assay also detects weak binding of MPA to α_1-acid glycoprotein, α_2-macroglobulin and IgM.

In the case of RCA the lectin-enzyme binding studies suggest that the lectin has a weak affinity for (fractions of) nearly all plasma glycoproteins, although the RCA lectin column retains only the ones with stronger affinities such as haptoglobin and IgA. Only Gc-globulin shows no affinity at all for RCA in the lectin-enzyme binding assay, apart of course from albumin. The results also indicate that a sufficient coating of the glycoproteins has been achieved. Thus, the observed low binding of a number of lectins for many glycoproteins is not due to a low coating efficiency. The results of WGA binding confirm this.

GalNAc was found by chemical techniques to occur linked to α_2-HS glycoprotein, IgA, 8Sα_3-glycprotein, β_2-glycoprotein III and IgD (Uhlenbruck et al., 1979). IgD was not tested in our system. We found binding of the lectins from Artocarpus integrifolia (Jacalin) and Artocarpus communis to α_2-HS-glycoprotein, IgA, Hemopexin, 8Sα_3-glycoprotein, AGP and α_2 macroglobulin. β_2-glycoprotein III did not bind. As these lectins appear to be GalNAc-specific, these correlations provide a validation of the lectin-enzyme binding studies, although some discrepancies remain.

Recently, affinodiffusion experiments in gels were done to investigate the

precipitation of plasma glycoproteins with a number of lectins (Dorner et al., 1986). The results are of interest in the light of our binding assays with the same lectins and glycoproteins. BPA did not precipitate with IgA and IgM, but it does with lactoferrin. The binding of MPA to IgA and AGP (weak) is confirmed, however no precipitation occurs with α_2M and IgM.

ECA precipitates with α_2M and haptoglobin, but our lectin-enzyme binding assays show binding to IgM only.

DSA precipitates only haptoglobin. The lectin-enzyme binding assay shows a strong reaction with haptoglobin, and a weak binding to six other plasma glycoproteins.

A number of conclusions may be drawn from our experiments and their comparison with other techniques.

First, except Gc-globulin, which shows very weak binding by a few one lectins, all other glycoproteins bind well to at least one lectin, and in some cases to many. Large differences in coating efficiency cannot therefore be the reason for the absence of binding of certain lectins to certain glycoproteins. Only in the case of Gc-globulin one might argue that the possibility of an exceptional low coating efficiency of Gc-globulin could be the reason for its lack of binding to all lectins. However, a more realistic explanation may be found in the very low carbohydrate content of Gc-globulin: it probably contains only one small glycan.

Second, the lack of binding of DSA which has a preference for biantennary asialo chains (Crowley et al., 1984) to most plasma glycoproteins indicates that few sialic acid groups if any were lost from the glycoprotein during its purification. This confirms remarks from Behringwerke who supplied the glycoproteins.

Third, the immunoglobulins appear to bind to many lectins to some extent. Indeed, immobilized IgG has even been advocated as a universally applicable column for the isolation of lectins (Franz et al., 1981), most of which will bind to the IgG column depending on its size, among other things. One of the reasons will be the heterogeneity in Fab and Fc glycosylation. This means that antibodies may not be used in lectin-enzyme immunoassays without one or more forms of pretreatment. One might consider chemical or enzymatic removal of carbohydrates, or adsorption of the binding fraction of the antibodies on columns containing immobilized lectins. Our preliminary studies (unpublished) indicate that these pretreatment measurements are not without problems. When antibodies can be prepared which do not contain carbohydrate or bind lectins, the lectin-enzyme immunoassays may be applied to many glycoproteins.

Fourth, the lectin-enzyme binding assay confirms results obtained with most other techniques to study lectin-glycoprotein interaction. However, weaker binding affinities are found using our assay which remain undetected by some other methods. A few reasons might be thought of.

1. Weak binding glycoproteins are retarded by lectin columns, but are still washed away by the buffer flow only. Depending on the volume of the unbound

fraction in relation to the column bed volume, they may either be found in the "unbound" or in the "bound" fraction, although they belong to neither.

2. Binding of lectins to glycoproteins does not necessarily lead to precipitation (Montreuil et al., 1983). This phenomenon still has to be explored in greater detail.

Fifth, the correlation between the binding patterns of LCA and PSA, and also of Jacalin and ACA validate aspects of the lectin-enzyme binding assay. These two pairs of lectins are both known to have similar binding specificities. The fact that they show comparable reaction patterns towards various glycoproteins coated in microtiter plates indicate that their purity, their way of isolation, their degree of biotinylation and the exact lectin-STAP complex composition are of very little influence on the observed lectin binding. These findings facilitate a more widespread use of lectin-enzyme binding assays.

Discrepancies between lectin-enzyme binding assays and lectin-column chromatography or lectin precipitation techniques could be due to the following:

- Impossibility to compare directly a lectin-column chromatogram pattern with absorbance measurements in the LEIA technique.
- Differences in glycoproteins in plasma and those subjected to an isolation procedure.
- Inability of some lectins to precipitate glycoproteins after binding (Glad et al., 1984).
- Uneven coating of glycoprotiens in microtiter plates (Butler et al., 1987).
- Different susceptibilities of various techniques for the number of glycans per molecule.
- An observed precipitation in gels or in solution is not easily quantified: it may be 10% or 100% of the glycoprotein.
- Differences in binding may exist between immobilized lectins and lectins in solution (Montreuil et al., 1983).

CHAPTER 5

APPLICATION OF VARIOUS FORMS OF LECTIN-ENZYME BINDING ASSAY TO INVESTIGATE THE GLYCOSYLATION OF IgG, HAPTOGLOBIN AND HCG

5.1. Introduction

To investigate the applicability of lectin-enzyme binding assays three proteins are selected of which disease-associated glycan modifications have been described recently. IgG from patients with rheumatoid arthritis contains less galactose (Parekh et al., 1985; Pekelharing et al., 1988). Haptoglobin from cancer patients shows increased fucosylation (Thompson et al., 1987), while HCG produced by tumors shows complex changes in glycosylation (Endo et al., 1987).

The three types of lectin-enzyme binding assays that we explored are different. IgG from RA patients was purified and passively coated to microtiter plates, after which lectin binding was studied. In the case of haptoglobin, its binding to hemoglobin coated in microtiter plates was exploited, followed by lectin binding studies. In this case the hemoglobin acts as the scavenger of haptoglobin from plasma, comparable with anti-haptoglobin antibodies, but it is not glycosylated. Only with HCG the lectin enzyme immunoassay variant was explored.

This chapter describes the three types of lectin-enzyme binding assays for IgG, haptoglobin and HCG, respectively.

5.2.1. *Alterations in carbohydrate composition and lectin binding of serum IgG from patients with rheumatoid arthritis and from pregnant women*

Immunoglobulin G is a major plasma glycoprotein containing, on average, 2.8 N-linked carbohydrate chains per molecule, of which 2 are located on the Fc fragment (Rademacher et al., 1984). The carbohydrate chains consist of a variety of diantennary complex-type structures, some containing an extra "intersecting" N-acetylglucosamine or a fucose residue.

The plasma of patients with rheumatoid arthritis (RA) contains aggregated immunoglobulins. These so-called "immune-complexes" differ from antigen-antibody complexes in several ways. Because of the absence of antigen it is suggested that the aggregates consist of certain immunoglobulins with affinity for other immunoglobulins (autoantibodies). The strength of the interaction between the immunoglobulins (Ka = 10^5 M^{-1}) is less than that observed for normal antigen-antibody interactions, indicating that a different type of interaction may be involved (Mannik et al., 1985).

The recent finding of a reduced galactosylation of the diantennary carbohydrate

chains of total IgG in patients with RA (Parekh et al., 1985) and a reduced B-cell galactosyltransferase (Axford et al., 1987) has led to a new hypothesis concerning the aggregation of IgG molecules. Less sugar constituents in the N-linked glycans of IgG could make IgG more "sticky" due to a lectin-like activity.

If incomplete glycosylation of IgG causes the formation of harmful IgG aggregates, then conditions inducing an increased plasma glycoprotein glycosylation, such as pregnancy (Raynes, 1982), could reverse this aggregation. A decrease in C1q-binding activity has indeed been found in RA patients during pregnancy (Pope et al., 1983), while a remission of RA during pregnancy is commonly observed (Ostensen et al., 1983; Persellin et al., 1981), indicating that the finding of an increased glycosylation of IgG during pregnancy would support the suggestion of a key role for the glycosylation of IgG in the pathogenesis of immune aggregates in rheumatoid arthritis.

To compare the carbohydrate composition of IgG samples from serum of pregnant women, patients with RA and healthy blood donors, detailed quantitative monosaccharide analysis by means of gas-liquid chromatography was performed.

Lectin binding to the purified IgG preparations passively adsorbed to microtiter plates was done as described in chapter 4.

5.2.2. *Materials and Methods*

1. Isolation of IgG
   Blood samples were obtained from pregnant women, healthy blood donors, and from patients with active RA visiting the rheumatology clinic. Serum was extracted with an equal volume of n-hexane to remove lipids, and dialysed for 24 h against 0.0175 M phosphate buffer, pH 6.3 (buffer A). After filtration, 2 ml serum samples were applied to a column (35 x 2 cm) of diethylaminoethyl-cellulose (DEAE) (Pharmacia, Uppsala, Sweden) equilibrated with buffer A. IgG was in the unbound fraction and eluted with buffer A. The bound fraction was eluted with 0.4 M phosphate buffer, pH 5.2, containing 2 M NaCl, and discarded. The column was regenerated by washing with 0.25 M phosphate buffer, pH 6.3 followed by buffer A.
2. Purity check
   Immunoelectrophoresis of the bound and the unbound fractions was carried out using a rabbit antiserum against human plasma proteins as well as specific rabbit antisera against IgG, IgA and IgM.
3. Sample preparation
   The isolated IgG was dialysed against distilled water for 3 days at 4°C. The samples were then freeze-dried and stored over P_2O_5 for 24 hours.
4. Monosaccharide analysis
   Carbohydrate samples were subjected to methanolysis (1.0 M methanolic HCl, 24 hours, 85°C) followed by gas-liquid chromatography of the trimethyl-silylated (N-reacetylated) methyl glycosides on a capillary CPsil5 WCOT fused

silica column (0.34 mm x 25 m, Chrompack, Middelburg, The Netherlands) (Kamerling et al., 1982).

5. Lectin-enzyme binding assay
   50 μl of a solution of 5 μg/ml of the purified IgG in PBS was transferred to microtiter plates and allowed to adhere for 4 hours at 37°C. Subsequently, plates were washed and the lectin binding studied as described in chapter 4.

5.2.3. *Results*

Immunoelectrophoresis of the unbound fraction with rabbit antiserum against human IgG showed only one large precipitation arc in the IgG region. With antiserum against human plasma proteins no other proteins were detected, in particular no IgA and IgM were detected. The bound fraction was found to contain all other plasma proteins together with a small amount of IgG (slides not shown).

The sugar analysis data of IgG obtained from ten blood donors, nine patients with RA and six pregnant women are given in table 5-1.
In table 5-2 the significance of the differences of the mean monosaccharide and sugar contents is given, calculated using the Mann-Whitney test.

The IgG from RA patients contained 20% less galactose than IgG from healthy subjects. The mean N-acetylglucosamine content was increased by 20%. The mean fucose and sialic acid contents were not different from normal. In pregnancy, the mean galactose content was increased by 13% and the mean sialic acid content by 44%, whereas N-acetylglucosamine and fucose contents were not different from normal.

The binding of one of the lectins (RCA) shows a relationship with the IgG carbohydrate composition (figure 5-1). Rheumatoid arthritis IgG binds significantly less of the galactose specific lectin. With the other lectins, galactose specific or not, no such relationship was found.

5.2.4. *Discussion and conclusions*

The monosaccharide analysis of IgG from patients with RA shows a significantly lower amount of galactose as compared to IgG from healthy individuals, whereas the N-acetylglucosamine content is significantly higher (Table 5-2). Although the analysis of carbohydrate content does not give information with respect to the glycan structure, it is reasonable to assume that the major difference between IgG from RA patients and normals is the reduced galactosylation of the diantennary glycans, with a concomitant increase in N-acetylglucosamine content. In an earlier study, only the under-galactosylation was indicated (Parekh et al., 1985). It was stated that there are no apparent changes in the levels of the β-N-acetylglucosaminyltransferase

Table 5-1. Monosaccharide composition per 3 mannoses and percent sugar of IgG from ten blood donors (N), nine patients with RA (RA) and six pregnant women (P).

	Man	GlcNAc	Gal	NeuAc	Fuc	% Sugar
N 1	3	3.09	0.79	0.20	0.70	2.9
N 2	3	3.08	1.03	0.24	0.73	2.1
N 3	3	2.94	0.74	0.14	0.72	2.1
N 4	3	3.26	0.83	0.21	0.76	2.2
N 5	3	3.29	0.87	0.16	0.71	2.4
N 6	3	3.13	0.75	0.13	0.69	2.2
N 7	3	2.93	0.82	0.22	0.67	2.5
N 8	3	3.30	0.93	0.20	0.77	3.0
N 9	3	2.91	0.71	0.18	0.66	2.3
N 10	3	3.01	0.88	0.14	0.63	1.9
Mean N	3	3.09	0.84	0.18	0.70	2.36
RA 1	3	3.50	0.61	0.14	0.73	2.2
RA 2	3	3.30	0.59	0.20	0.74	2.2
RA 3	3	3.49	0.59	0.14	0.74	2.4
RA 4	3	3.24	0.57	0.12	0.71	2.0
RA 5	3	3.30	0.58	0.14	0.72	2.1
RA 6	3	3.19	0.62	0.11	0.68	1.8
RA 7	3	3.23	0.92	0.28	0.63	1.9
RA 8	3	3.24	0.69	0.13	0.78	2.0
RA 9	3	3.32	0.88	0.21	0.68	1.9
Mean RA	3	3.31	0.67	0.16	0.71	2.06
P 1	3	3.23	0.95	0.26	0.84	2.1
P 2	3	3.18	0.98	0.21	0.64	3.5
P 3	3	3.15	0.77	0.23	0.71	2.1
P 4	3	3.16	0.96	0.22	0.65	2.1
P 5	3	3.14	1.10	0.24	0.70	2.3
P 6	3	3.17	0.94	0.23	0.70	2.3
Mean P	3	3.17	0.95	0.23	0.71	2.40

Man : Mannose; GlcNAc: N-acetyl-glucosamine; NeuAc : N-acetyl-neuraminic acid; Fuc : Fucose.

enzymes GnT I, GnT II, GnT III and $\alpha(1\rightarrow6)$-fucosyltransferase. Taking into account that only diantennary structures were detected (Parekh et al., 1985), the observed increase of N-acetylglucosamine in this study has to be explained as a change in GnT III activity, responsible for the attachment of the intersecting N-acetylglucosamine. The presence of this sugar residue reduces the galactosylation of the Man $\alpha(1\rightarrow3)$-arm by about 78% (Narasimhan et al., 1985). Thus, if the 20% increase of N-acetylglucosamine residues are of the intersecting type, this would result in a decrease of galactose residues of about 16%, comparable with the 20% that we found. Taken together, our results suggest

Table 5-2. The mean monosaccharide content (per 3 mannoses) and sugar content (%), standard deviations and significance of the differences of IgG from healthy blood donors (n = 10), patients with RA (n=9), and pregnant women (n = 6).

	Blood donors	RA patients	P Value
GlcNAc	3.09 ± 0.15	3.31 ± 0.11	$p < 0.01$
Gal	0.84 ± 0.10	0.67 ± 0.13	$p < 0.05$
NeuAc	0.18 ± 0.04	0.16 ± 0.06	NS
Fuc	0.70 ± 0.04	0.71 ± 0.04	NS
Sugar (%)	2.36 ± 0.35	2.06 ± 0.19	$p < 0.05$
	Blood donors	Pregnant women	
GlcNAc	3.09 ± 0.15	3.17 ± 0.03	NS
Gal	0.84 ± 0.10	0.95 ± 0.11	$p < 0.05$
NeuAc	0.18 ± 0.04	0.23 ± 0.02	$p < 0.05$
Fuc	0.70 ± 0.04	0.71 ± 0.07	NS
Sugar (%)	2.36 ± 0.35	2.40 ± 0.55	NS
	Pregnant women	RA patients	
GlcNAc	3.17 ± 0.03	3.31 ± 0.11	$p < 0.01$
Gal	0.95 ± 0.11	0.67 ± 0.13	$p < 0.01$
NeuAc	0.23 ± 0.02	0.16 ± 0.06	$p < 0.05$
Fuc	0.71 ± 0.07	0.71 ± 0.04	NS
Sugar (%)	2.40 ± 0.55	2.06 ± 0.19	NS

Values are mean ± SD. NS: not significant: $p > 0.05$; Mann-Whitney test

a 20% increase in intersecting N-acetylglucosamine residues resulting in under galactosylation of IgG in patients with RA. Direct measurement of B-cell galactosyltransferase indicates lower enzyme activity in RA patients (Axford et al., 1987). Other recent studies also point towards abnormal glycosyltransferase activities in autoimmunity (Imai et al., 1988).

Using a solid phase lectin binding assay, others found that IgG from patients with RA bound significantly more to the immobilised plant lectins Peanut agglutinin (PNA) and Concanavalin A (ConA) than IgG from normals (Malaise et al., 1987).

This indicates either an increased content or an increased availability of terminal galactose groups (binding to the PNA lectin) and diantennary sugar chains (binding to the ConA lectin). As our results and those obtained by others (Parekh et al., 1985) indicate a decreased galactose content and suggest a decreased Con A binding owing to the intersecting GlcNAc, a conformational change in the structure of IgG in patients with RA leading to increased lectin binding could explain these results. Indeed, an altered conformation of IgG in RA has recently been suggested (Hanson et al., 1985).

Our findings could support a viral aetiology of the disease. Viruses may alter the glycosylation of cell glycoproteins (Groen, 1984; Duc Dodon et al., 1984; Avango et al., 1988). If the IgG producing B-cells are infected by a

virus, for instance the RA associated Epstein-Barr virus (Yao et al., 1986) which has a preference for B-lymphocytes (Frade et al., 1985), the result could be a modified glycosylation of IgG produced by the infected cells. This mechanism has been shown to occur in lymphoblastoid cells infected by influenza virus, where decreased glycoprotein sialylation takes place and is only partially compensated for by an increased cellular sialyltransferase (Duc Dodon et al., 1984). Another example is the polyoma virus transformation of baby hamster kidney cells, which induces a twofold increase in the activity of the enzyme GnT V that adds N-acetylglucosamine β1-6 to the α1-6 linked mannose (Yamashita et al., 1985). Quite recently the lowered galactosyltransferase of B-cells has indeed been found in RA patients (Axford et al., 1987). An alternative explanation suggests that mycobacterial antigens are involved in the development of RA (Shoenfeld et al., 1988; Rook et al., 1988).

If a reduced galactose content of IgG is a prerequisite for the appearance of (some of) the symptoms associated with RA, conditions of increased galactosylation could form a compensation and could be the basis of a therapeutic approach. Such a condition may exist for IgG during pregnancy or estrogen therapy, two processes where an increased glycosylation of other plasma glycoproteins has been found (Raynes, 1982).

Our results show that a significant increase (13%) occurs of the galactose content of IgG during pregnancy. The galactose content of IgG in pregnancy is 42% higher than in the RA group. Thus, a partial compensation during pregnancy of the IgG under galactosylation in female patients with RA can be imagined. IgG from one female RA patient during pregnancy was analysed (results not shown). It contained high levels of galactose as found in the pregnancy group, and also high levels of N-acetylglucosamine as found in the RA group. Owing to the slight variation in carbohydrate analysis within each group this result has to be confirmed by carbohydrate analysis of IgG obtained from a larger number of pregnant women with RA.

Together with an increase of IgG galactose during pregnancy an increase of sialic acid is also found. This suggests a more complete glycosylation of the diantennary glycans of IgG during pregnancy. The N-acetylglucosamine content of IgG during pregnancy is not different from normal.

Given the fact that the half-life of IgG in the circulation is approximately three weeks, the recurrence of symptoms in female patients with RA a few weeks after delivery can also be explained, because the more galactosylated and sialylated IgG produced during pregnancy will gradually be replaced by the RA-associated undergalactosylated IgG with possible aggregating properties produced thereafter. Alternatively, the increased N-acetylglucosamine content of RA IgG could also have a role, but no decrease has been found to occur during pregnancy.

The changes in IgG glycosylation in pregnancy may be due to the altered hormonal state. Estrogens (Raynes, 1982; Starr et al., 1985) as well as prolactin (Bradshaw et al., 1985) influence glycoprotein glycosylation. The association

between autoimmune diseases such as RA and steroid hormones has been well described (Ahmed et al., 1985; Masi et al., 1984). Oral contraceptives (Van den Broucke et al., 1982, 1986) as well as the number of pregnancies (Silman, 1986) also have an influence. These results suggest that the glycosylation of IgG may play a role in the occurence of symptoms in RA. The differences between infusion therapies with placental IgG and serum derived IgG could thus be explained (Sany et al., 1987).

Studies with asialylated IgG prepared in vitro underline the relation between the IgG glycosylation and rheumatoid factors (Duc Dodon et al., 1981; Galloway et al., 1983). It would be of interest, in view of our results mentioned above, to extend these experiments with asialo-agalacto IgG prepared in vitro. Other studies have shown that differences in glycosylation of one Fab arm exist between precipitating and non-precipitating antibodies (Leoni et al., 1986; Labeta et al., 1986). Thus, the characteristics of IgG are delicately dependent on the glycosylation of the molecule.

The fact that remission of the symptoms often occurs in women with RA during pregnancy, together with our finding of an increase in IgG galactosylation in healthy pregnant women, support the view (Parekh et al., 1985) that IgG glycosylation have play an important role in the disease. The carbohydrate analysis of IgG of female patients with RA during pregnancy and the investigation of aggregating characteristics of asialo-agalacto IgG prepared in vitro could clarify this point.

A relationship between carbohydrate composition of IgG and lectin binding was obtained with only one of the lectins (RCA). As shown in figure 5-1 the RCA binding gives a good representation of the galactose content of IgG.

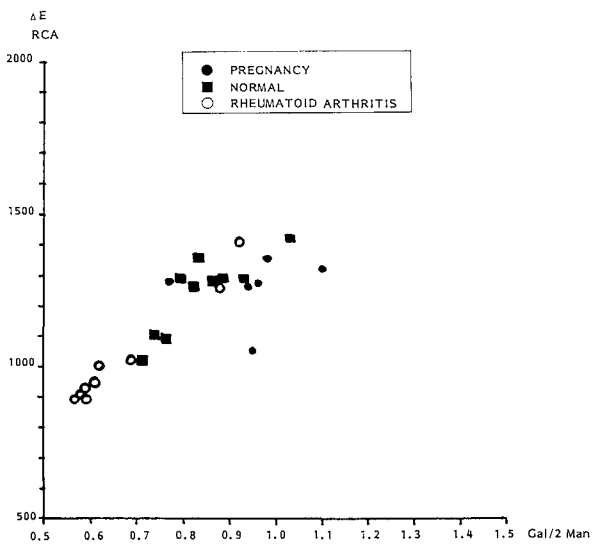


Figure 5-1. Relationship between RCA binding to microtiter plate wells coated with IgG and the IgG galactose content.

The reason why RCA is the only binding lectin may be found in its broad specificity with respect to asialo glycoproteins, as discussed in chapter 1.

Summarising, our results show that in principle quantification of IgG galactose content is possible using the lectin-enzyme binding assay. Our assay employed passive adsorption of purified IgG. The further development of the assay should focus on the pretreatment of an IgG-catching "first" antibody or Protein A coating of the solid phase in such a way that no binding of RCA lectin occurs in the absence of sample. One might think of enzymatic pretreatment (such as N-glycanase or Endoglycosidase) or chemical pretreatment. Our results also indicate that with a glycoprotein purified from various patients it is possible to select the lectin which is able to make a distinction between healthy persons and patients.

All lectins tested show binding to the coated IgG to a greater or smaller extent. This means that high background binding is to be expected with antibodies to the glycoprotein under study, even when they are from quite different species such as rabbit, goat or sheep. While this may seem on first sight a serious flaw of the concept of lectin-enzyme immunoassays, it appears that in some cases the binding of the lectin-STAP conjugate to the coated antibodies is inhibited by the presence of the antigen in question. This observation would obviate the use of affinity-purified or monoclonal antibodies. In the development of the lectin-enzyme immunoassay for HCG glycovariants (chapter 5.4) the phenomenon described above was exploited succesfully.

5.3.1. *Lectin-enzyme binding assays detect disease-associated changes in glycosylation of serum haptoglobin bound to hemoglobin coated microtiter plates*

While studying the feasability of lectin-enzyme immunoassays the problem of binding of the lectin to the coated antibody, leading to high background values, was encountered. A number of steps were taken in an attempt to lower the background binding such as N-glycanase treatment of the antibodies, but so far they have been without effect.

A solution may possibly be found in the use of monoclonal antibodies raised in vitro in the presence of glycosylation inhibitors. At present, however, the binding of lectins to the carbohydrate determinants of the coated immunoglobulins remains a problem to be solved.

However, if one wishes to investigate the application of lectin-enzyme binding assays with serum samples in the clinical practice without using antibody coating, the affinity of haptoglobin for hemoglobin could be exploited. Hemoglobin is a non-glycosylated intracellular protein found in erythrocytes, which forms a stable complex with extracellular haptoglobin when hemolysis occurs.

Haptoglobin occurs in three genetic phenotypes: Hp 1-1, Hp 2-1 and Hp 2-2. The basic structure of haptoglobin is a four-chain molecule with two α- and two β-chains. Haptoglobin 1-1 contain α_1 chains, while haptoglobin 2-2 contains

α_2 chains. Both α_1 and α_2 chains are present in haptoglobin 2-1. The β-chain contains the oligosaccharide units: eight asparagine-linked carbohydrate chains; four of them being biantennary and four being triantennary branches. Analysis of intact human haptoglobin revealed virtually identical amounts of carbohydrate on all three subsets.

Modifications of haptoglobin glycosylation have been demonstrated recently in sera of patients with cancer (Thompson et al., 1987) using lectin-column extraction followed by SDS-PAGE and Western blots or silver staining. To investigate serum haptoglobin glycosylation with lectin-enzyme binding we developed the assay based on hemoglobin-coated microtiter plates.

5.3.2. *Materials and Methods*

Hemoglobin (type IV, Sigma, St. Louis) was dissolved in 0.1 M carbonate buffer pH 9.5 containing 0.2 g/l sodium azide, to a concentration of 50 μg/ml. Coating was accomplished by incubating 100 μl of the solution in microtiter plate wells at room temperature for 24 hours. Five washings with PBS-Tween (0.05%) followed. Dried plates were stored in aluminium foil at -20°C.

Haptoglobin concentrations in serum samples were determined using a Behring Nephelometer Analyser (BNA) with Behring antiserum according to the manufacturers instructions. Samples were diluted with PBS-Tween to a haptoglobin concentration of 50 μg/ml. 100 μl diluted serum was allowed to interact with the hemoglobin-coated wells for 2 hours at 37°C, followed by five washings with PBS-Tween.

The lectin-enzyme binding assay was done as described in chapter 4. As a buffer Tris-buffered saline (TBS) was used with the following composition: Tris 100 mM, NaCl 100 mM, $MgCl_2.6H_2O$ 0.5 mM, $CaCl_2.2H_2O$ 1.0 mM. Set pH at 8.0 with 1.0 M HCl, add $MnCl_2.4H_2O$ 0.1 mM. Washings were done with TBS-tween (0.05%). Incubations of lectin-STAP complex were done at 4°C. All assays were done in duplicate.

Serum samples were used from patients with cancer showing an elevated tumor marker, such as CEA (n=8), AFP (n=2), paraprotein (n=7) or prostatic acid phosphatase (n=5), and from normal blood donors (n=4).

In a separate experiment differences of lectin binding to haptoglobin were determined for six lectins and ten normal serum samples.

5.3.3. *Results*

The mean extinction in the lectin-enzyme binding assay of the control samples (blood donors) is set at 1 for each lectin. For the individual cancer sera, results are given in table 5-3 and expressed as the extinction found divided by the mean extinction of the control groups. The differences between the lectin binding assays of the normal blood samples (n=10) proved to be small. The most extreme values are given at the bottom of table 5-3.

Table 5-3. Extinctions in haptoglobin lectin-enzyme binding assays, expressed as times the mean of normal.

Patient	Elevated tumor marker	LCA	WGA	BPA	AAA	Lotus	AAL
1	CEA	1.0	1.4	0.8	0.9	1.6	1.0
2	CEA	1.0	1.6	0.6	0.8	1.8	1.0
3	CEA	1.3	2.2	1.2	1.2	2.3	1.2
4	CEA	0.8	2.4	0.6	0.9	2.0	1.1
5	CEA	0.9	0.8	1.0	0.7	1.0	1.3
6	Au+	1.2	1.6	1.2	0.9	1.5	1.5
7	Au+	1.2	2.0	1.0	1.0	1.6	1.6
8	IgAk	0.7	2.4	0.8	0.8	1.7	1.1
9	IgGλ	0.6	1.8	0.8	0.8	1.1	1.0
10	IgGλ	0.9	1.2	0.7	0.6	0.7	0.9
11	IgAk	0.7	1.5	0.7	0.7	1.3	1.3
12	IgGk	1.0	3.7	0.9	1.0	2.7	1.4
13	IgGλ	0.7	3.0	0.7	0.9	2.1	0.9
14	IgMk	0.9	3.3	0.8	1.0	1.8	1.5
15	PZF	0.8	1.6	0.9	0.7	1.7	0.9
16	PZF	0.7	1.8	0.6	0.6	2.0	0.9
17	PZF	0.7	1.0	0.5	0.4	1.2	1.0
18	PZF	0.9	1.4	0.8	0.6	1.2	1.5
19	PZF	0.6	4.0	0.9	1.0	3.7	0.9
20	CEA	1.2	1.0	1.0	11.3	1.6	0.5
21	CEA	1.1	2.8	4.6	1.3	1.7	3.9
22	CEA	1.7	>4.0	1.7	1.8	3.1	1.6
Normal range (n=10)		0.7-1.3		0.7-1.2		0.7-1.3	
(extremes)			0.9-1.1		0.8-1.2		0.6-1.4

5.3.4. *Discussion and conclusions*

In most cancer patients our lectin-enzyme binding assay detects cancer-associated changes in serum haptoglobin glycosylation (table 5-3).

Using six lectins, 4 out of 22 cancer sera showed abnormal binding of one lectin, 15 sera showed increased binding of two lectins. One serum sample did not show abnormal haptoglobin glycosylation as tested with the lectins used. This does not imply, however, that no change in haptoglobin glycosylation occurs in these patients, because the glycosylation of haptoglobin also affects the binding of haptoglobin to hemoglobin (Katnik, 1984). Thus, if aberrantly glycosylated subfractions of haptoglobin that do not bind hemoglobin are present in serum, they will also not bind to the hemoglobin-coated wells of the microtiter plate and remain undetected in this system. Such fractions can only be quantitated using antihaptoglobin coated microtiter plates, assuming that the glycosylation does not have an influence on the binding of antibodies. The inability of certain

haptoglobin subfractions to bind hemoglobin could explain the persistence of haptoglobin in the circulation of some patients with a proven chronic hemolysis.

The increase in the aberrantly glycosylated haptoglobin subfraction is higher than the factor of the ratio described above. In ELISA systems an increase in extinction with a factor 4 may correspond to an increase in concentration with a factor 100. Thus, the observed changes in haptoglobin are much more pronounced than the extinction ratio would suggest.

The mechanism behind the abnormal plasma haptoglobin glycosylation remains unclear. Synthesis and excretion by tumors of aberrantly glycosylated haptoglobin has not yet been described (except in case of primary liver cell carcinoma), and if it occurs its contribution to the plasma haptoglobin concentration will remain extremely small. It is more likely that certain active factors have an influence on the liver, just as monokines have on acute phase protein synthesis, resulting in the appearance of unusual subfractions of haptoglobin.

Low-grade hemolysis in the tumor leading to the accumulation of subfractions not capable of hemoglobin binding may also occur, but these fractions are probably not detected in a hemoglobin-based assay such as the one described above.

A few patients haptoglobins show extremely elevated binding of one or two of the lectins (patient 19 with WGA and Lotus, patient 20 with AAA, patient 21 with BPA and patient 22 with WGA). The reason and significance of these findings remain to be clarified.

Two lectins out of the six tested show by far the greatest discriminating capacity, both highly binding to fifteen out of the twenty-two cancer sera haptoglobin. These results confirm those obtained with lectin affinity chromatography with one of these two lectins (Lotus), followed by 1D or 2D PAGE and silver staining, where increased binding of haptoglobin in sera from cancer patients was also noted (Thompson et al., 1987). The increase of serum fucose content in cancer (Waalkes et al., 1983) could be caused by elevated levels of highly fucosylated haptoglobins.

The column technique also detects increased Lotus binding of haptoglobin from rheumatoid arthritis sera, although the apparent molecular weight of these haptoglobins in SDS-PAGE is different from cancer sera haptoglobin. Our most recent results (not shown) with the lectin-enzyme binding assay confirms increased binding of Lotus lectin to RA haptoglobin. However, no increased WGA binding was found as with cancer sera haptoglobin, while highly increased MPA binding was found which does not occur with the cancer samples. Taken together, using a panel of lectins both mutual and different glycosylation modifications in haptoglobins from cancer patients and from rheumatoid arthritis patients may thus be detected.

There appears to be a linkage between WGA and Lotus binding. This suggests increased sialic acid content (increased branching and terminal glycosylation, a common finding in cancer) leading to increased WGA binding, and increased fucose incorporation (not uncommon in cancer as well) leading to more Lotus binding. The fucose is probably located at the branches of haptoglobins glycans,

as LCA (binding to biantennary glycans with α1-6 at the base) does not show increased binding. Any relationship with the haptoglobin subtypes, although highly unlikely, remains to be investigated.

In conclusion, the lectin-enzyme binding assay detects certain aberrantly glycosylated subfractions of haptoglobin bound to hemoglobin-coated microtiter plates in patients with cancer, as well as certain other diseases. Detailed clinical studies are necessary to elucidate the significance of these findings.

5.4.1. *Malignancy-associated modifications in serum HCG glycosylation are detected by lectin-enzyme immunoassays*

HCG contains two N-linked glycans at aminoacids 52 and 78 in its α-chain and two in its hormone-specific β-chain at positions 13 and 30. Compared with LH and FSH, the β-chain of HCG has an extra peptide which contains four O-linked glycans at aminoacids 121, 127, 132 and 138. The α-chain of HCG produced by a tumor has an increased content of α1-6-fucosylated biantennary sugar chains, and appears to possess the unusual α1-6-fucosylated tri- and tetra-antennary chains. Aberrant phosphorylation may also occur (Cox, 1986; Saccuzzo et al., 1986; Endo et al., 1987; Kobata, 1988; Iwase, 1988). Attempts have been made to quantitate these changes by lectin affinity chromatography (Koyama et al., 1986; Cole et al., 1984; Nishimura et al., 1985; Chapman et al., 1984), by chemical analysis of the HCG-linked sugar chains (Cole, 1987; Mizuochi et al., 1983) or by lectin-immunoradiometric assay (Imamura et al., 1987).

We investigated the feasability of lectin-enzyme immunoassays with a number of lectins to see if the assays are capable of distinguishing HCG produced by the placenta and by tumors, as well as HCG produced by hydatidiform mole. This latter category is of especial interest as an invasive mole may transform into a trophoblastic tumor.

The results indicate that with certain lectins this distinction can be made using lectin-enzyme immunoassays.

5.4.2. *Materials and Methods*

- Samples were obtained from Nijmegen University (Dr. Idema) and from the Daniel den Hoed Cancer Clinic Rotterdam (Dr. Haije).
- Lectins: the lectins used are described in table 4-1.
- HCG determination.
  HCG was determined with a RIA technique using a kit from Diagnostic Products Corporation, Los Angeles, USA.
- Lectin-enzyme immunoassay
  100 μl monoclonal anti-HCG clone 293A (Sanbio, Uden, Netherlands) diluted in carbonate buffer was allowed to coat microtiter plate wells at 37°C for 4 hours. Plates were then washed 4 times with PBS-tween. Plates were incubated

with 50 μl HCG containing sample, diluted to 200 U/L, at 37°C for 2 hours. After washing the plate four times the lectin-enzyme binding was allowed to take place as described in chapter 4.

5.4.3. *Results*

Table 5-4 gives the extinctions in anti-HCG coated wells incubated with HCG containing serum samples from tumor patients (T), molar pregnancy (M) and healthy pregnant women (N).

5.4.4. *Discussion and conclusions*

All lectins bind to some extent to the coated monoclonal anti-HCG in the absence of sample ("blank binding" in table 5-4). The presence of 10 U HCG in the well decreases the lectin binding in most cases. This is probably an effect of steric hindrance: HCG diminishes the accessibility of the lectin for the immunoglobin glycans when HCG is captured. If the HCG molecules do not possess an equal amount of similar glycans, a decrease in binding may be observed. Only in the case of RCA an equal and increased lectin binding is observed in the presence of HCG. Any role of the specificity of the anti-HCG antibody remains to be established.

We calculated the mean $\pm$ two standard deviations of the extinction coefficients for each lectin in the control group (n=11). Out of these six limits (for six lectins) one tumor patient exceeded two, one exceeded three, one exceeded four and one exceeded six limits, roughly correlating with the HCG concentrations in the original (undiluted) samples. Three out of four tumor patients were outside these ranges with respect to binding to the lectins RCA, LCA, Ulex and PNA. Two patients showed aberrant AAL binding, and one aberrant AAA binding.

With respect to the increased fucose lectin-binding these results are consistent with the known modifications of HCG glycosylation in patients with HCG producing tumors (Mizuochi et al., 1983; Imamura et al., 1987; Endo et al., 1988; Kobata, 1988). Increased fucosylation may lead to increased binding of one or more of the fucose-specific lectins LCA, Ulex 1, AAL, Lotus and AAA, depending on the branching pattern and the attachment site and linkage of fucose. All of these lectins show increased binding to one to three, out of the four HCG's from tumor patients.

However, while increased RCA and PNA binding was found before by a LIRMA technique (lectin immunoradiometric assay), we found decreased RCA and PNA binding, indicating a decrease in terminal Galβ1-4GlcNAc and Galβ1-3GalNAc structures, respectively. The reason for this discrepancy remains unclear at present. Inhibition by other glycoproteins present in the urine may play a role, because the LIRMA technique, being comparable to our type I lectin-enzyme immunoassay (chapter 2), will show the same drawbacks such as crossreactivity by different proteins with comparable glycans.

Table 5-4. HCG concentrations and lectin binding in patients with tumor (T), mole (M) compared with healthy pregnant subjects (N) with mean, σ and limits expressed in milli-extinction units.

		LECTINS						
Blank Binding	HCG U/l	RCA 245	LCA 270	Ulex 1 610	AAL 1010	PNA 215	AAA 475	Score
T1	1.417	291	94	312	485	55	193	4
T3	442	316	180	230	679	162	185	3
T4	89.000	252	144	400	774	98	264	6
T5	434	298	60	133	343	91	128	2
M1	19.280	294	100	150	471	67	170	3
M2	19.640	318	97	283	534	92	210	4
M3	32.780	293	50	134	382	76	264	3
M4	136.820	262	76	114	431	100	157	1
M5	109.330	275	75	232	510	88	200	4
M7	245.000	271	54	117	460	112	266	2
M8	10.200	348	58	72	329	88	134	1
N1	15.900	394	98	216	515	116	195	2
N2	28.000	356	69	110	376	129	170	0
N3	95.000	314	55	82	444	114	142	0
N4	11.000	397	70	165	395	130	143	0
N5	30.000	340	50	128	378	155	258	1
N6	35.000	342	51	82	396	111	163	0
N7	13.140	406	64	184	450	153	166	0
N8	32.400	352	46	146	377	159	190	0
N9	56.830	342	56	94	372	129	124	0
N10	21.410	402	56	114	467	162	138	0
N11	8.320	401	68	188	406	186	206	1
T		290	120	268	570	100	190	
σ		25	46	100	170	40	47	
M		294	70	157	445	89	200	
σ		25	20	68	66	14	47	
N		370	62	137	416	140	170	
σ		31	13	44	44	20	36	
Limits $X \pm \sigma$		308- 432	36- 88	49- 225	328- 504	100- 180	98- 242	

Underlined: values outside limits.
Score: Number of lectins (out of six) with abnormal binding.

Of the six patients with molar pregnancy two showed binding outside four limits, two outside three limits, one outside two limits and two outside one limit. Here, both RCA and PNA detected five out of seven patients, while Ulex, AAL and AAA detected two.

Of the normal samples one had two values (out of six) out of the mean $\pm$

2 SD range. These lectins were LCA and AAL both with affinity for biantennary glycans with a α1-6 linked fucose in the core. The significance of this finding is unknown at present. A theoretical explanation could be found in the gestation time dependent glycosylation (as is the case with AFP glycosylation). HCG early in pregnancy could very well be glycosylated differently from HCG produced in later trimesters. It is unknown whether the first normal sample was taken at a different moment in pregnancy as the others (all were blood group Rhesus control samples). Two other normal samples out of the remaining ten showed binding of one lectin outside the limits.

There appears to exist no relationship between the HCG concentration and the extent of aberrant glycosylation, that is, the number of lectins highly bound, or the extinction measured per lectin, in the molar and normal pregnancy groups.

The HCG samples were diluted to an equal concentration of 200 U/l. This concentration was considered to be sufficient to saturate the antibody coating with HCG. This is supported by the fact that in the patient categories both lower and higher lectin binding were observed.

Interference of rheumatoid factors is also excluded by the large dilution factors used. Besides, rheumatoid factors are rare in the subject categories studied.

In conclusion, the lectin-enzyme immunoassays of HCG appear to be able to characterise the modifications of HCG glycosylation in patients with hydatidiform mole or persistent trophoblastic disease, who may ultimately develop HCG producing choriocarcinoma. The assay will be of value in the understanding of the mechanisms of changes in protein glycosylation and the relationship with malignancy. The assay will also be of value in the diagnosis and treatment of patients in the above categories. However, differences between patients may occur as well as in one patient during the disease (Endo et al., 1988).

CHAPTER 6

GENERAL DISCUSSION

Glycoproteins in human plasma show disease-associated modifications of their carbohydrate chains. No systematic change has been found: hypo- or hyper-sialylation, increased branching or fucosylation have been described. Until now, fast, sensitive and specific methods to characterise glycan structures were lacking. The present work describes the development and application of solid phase enzyme binding assays employing lectins to study protein glycosylation. The sensitivity, specificity and speed of such assays was considered a major advantage over classical carbohydrate analysis and other techniques.

From the experiments described in chapter 2 it is concluded that the type I assay using an immobilized lectin and enzyme-linked antibodies will have few applications. The selectivity of the lectins tested is considered insufficient due to the possible binding of other glycoproteins to the coated lectin. Still, this technique may be applied to purified glycoproteins where the problem of cross-reactivity does not exist. One might even think of heterologeous lectin-enzyme assays which quantitate the binding of two lectins to the glycoprotein (Kohn, 1983). This type of assay was not further developed by us.

Another drawback of the type I assay could be the low affinity of the immobilized lectins to the soluble glycoprotein, depending on the average intramolecular distance between lectin molecules relative to the size of the glycoprotein in question. When the affinity is low due to the rigidity of the lectin surface the glycoprotein will be washed away during wash cycles.

In the type II assay immobilized antibodies are used which do not let their antigen go during washing cycles, and a lectin-STAP complex is used which probably has a far greater flexibility to overcome spatial restrictions in the interaction between (biotinylated) lectin molecules in the lectin-STAP complex and antibody-captured glycoprotein glycans. In any case the binding of lectin-STAP complexes to solid phase bound glycoproteins or glycoconjugates is firm enough not to be disrupted during wash steps.

To study the binding of lectins to immobilized glycoproteins and to investigate the dependence of the interaction on the number of interacting carbohydrate groups it was necessary to prepare well-defined glycoprotein subfractions. The experiments in chapter 3 indicate that the preparation and separation of the five transferrin sialovariants could be achieved. This is probably one of the very few glycoproteins allowing easy separation on the basis of charge as the result of differences of one sialic acid residue. Thus, for other glycoproteins it may be more complicated to prepare well-defined subsets. However, high-resolution ion-exchange chromatography or high-pressure lectin-column chromatography

may provide a solution. In any case, the human transferrin sialovariants are easily and completely separated from each other by high-resolution FPLC techniques.

With these five available fractions, aspects of the type II lectin-enzyme immunoassay using immobilized antibodies and an enzyme-bound lectin were investigated in detail (chapter 3). A standard curve was obtained with these well-defined sialovariants bearing whole integers of terminal galactose residues. Thus, while the response in the sandwich ELISA is a function of the amount of glycoprotein recognized by the enzyme-linked antibody, the response in the lectin-enzyme immunoassay is a function of the amount of glycan structure recognized by the enzyme-linked lectin. In the case of transferrin and RCA lectin results may be expressed as the amount of galactose residues per transferrin molecule.

Standardization is less simple when a glycoprotein has more carbohydrate chains. In a number of instances it will prove to be impossible to isolate or prepare subfractions or glycovariants of the glycoprotein in question. This approach would have the drawback that the standard material is different for each lectin, which does not favor the simultaneous binding studies of a large number of lectins. In such cases it may be possible to assign an arbitrary value to a large pool of standard material of undetermined composition from which individual laboratories may obtain small amounts. This is not unlike the WHO serum samples, for instance for immunoglobulins. Results are expressed in International Units per liter. Another approach would be to determine the reference range each time the assay is performed, by inclusion of a number of normal samples in the analysis. This approach has been followed in our HCG and haptoglobin studies. Results are then expressed in terms of the reference range.

It should be noted here that the method described may not be directly compared with other techniques such as lectin affinity chromatography or crossed affino-immunoelectrophoresis. Our method gives an overall impression of binding of the lectin used to the total amount of the glycoprotein present in the sample. The other methods allow the investigation of non-binding, weakly binding and strong binding fractions all at the same time. This could be achieved in the lectin-enzyme binding assay by adding serial dilutions of a competing sugar as described in chapter 4. This area was not further investigated.

In most cases lectins do recognize an "epitope" of more than a terminal or internal sugar group alone. Other groups present in the carbohydrate chain, sometimes located at a relatively large distance, may have an influence, sometimes decisive, on the strength of the binding interaction. Thus, while the method described may be very suitable to detect differences in binding, it is at present not interpretable in such a way that conclusions with respect to the exact carbohydrate structures can be drawn. When the molecular aspects of glycan modifications are known, this drawback will lose importance. On the other hand, the lectin-enzyme binding assays proved to be capable of detecting glycan modifications in disease (chapter 5), irrespective of the exact molecular basis

of the altered lectin binding. In the near future fundamental structural work will no doubt uncover the changes in the responsible glycosidases and glycosyltransferases (as is the case in rheumatoid arthritis), which lead to the occurrence of aberrantly glycosylated glycoproteins in the circulation during disease.

Classically, the specificity of lectins has been determined by inhibiting the lectin-induced hemagglutination with well-defined sugars. Our lectin-enzyme binding assay could be a new alternative, provided that the binding of the lectin-enzyme complex can also be inhibited by simple sugars in a concentration dependent manner. The experiments described in chapter 4 show that this is indeed the case. The method opens the way to characterize lectin specificities in more detail. Macromolecules such as glycoproteins, neoglycoconjugates and polysaccharides may also be used. A major advantage is the consumption of only extremely small amounts of lectin (typically 0.1 μg per assay). Another advantage is the unequivocal representation of the results in terms of 50% inhibition concentration.

In order to compose a lectin-STAP complex consisting of equivalent amounts of biotinylated lectin and streptavidin-peroxidase, a new method was developed as mentioned in chapter 4.3. The use of avidin coated plates as well as albumin-biotin coated plates allows the determination of the required lectin-biotin dilution and the required streptavidin-peroxidase solution. If during binding experiments in microtiter plates, wells coated with avidin and albumin-biotin are introduced next to the classical non-coated blank wells, one always has a check if the lectin-STAP complex was of the right composition. In all our subsequent experiments with plates coated with BSA-glycoconjugates and plasma proteins as well as the studies with patient samples we reserved three wells for avidin coating and three wells for albumin-biotin coating. We observed no change in optimal dilutions during that period with any of the lectins used.

After having determined the desired composition of a number of lectin-STAP complexes, the binding to well-defined neoglycoproteins adsorbed in microtiter plates was investigated. This is a new approach employing glycans of extended size. The lectin-enzyme binding assay detected large differences in binding of the lectins to the various structures. These were generally in accordance with results obtained with hemagglutination or lectin column techniques. Our method will gain importance when more BSA-glycoconjugates become available, allowing the simultaneous investigation of the binding of a large number of lectins to a large number of BSA-glycoconjugates. In a later stage the method might be used to select lectins for binding to well known structures, as well as to investigate unknown structures with a panel of lectins.

In the same way as described above, the binding of lectin-STAP complexes was determined in microtiter plates coated with purified plasma glycoproteins. From these studies the background binding in lectin-enzyme binding assays may be deduced, which may be of value when selecting the lectin to be used. Also, the binding of a large panel of lectins will ultimately lead to more insight into the glycan structures of glycoproteins when the specificities of the lectins are

known in detail. It is speculated that computerized processing of the results will be a necessity.

Clinical applications of the developed assay were investigated with respect to three plasma glycoproteins: IgG, haptoglobin and HCG.

In the case of IgG, passive coating of the purified protein from rheumatoid arthritis patients, pregnant women and blood donors was used, followed by lectin binding studies. In general, this method is applicable for each plasma protein. However, the purification procedures are time consuming and cumbersome. Besides, it has not yet been established that during purification of a glycoprotein all its subfractions are obtained with the same yield. When using ion-exchange chromatography, for instance, and applying narrow cut-off values to increase the purification factor, one selectively dismisses the extremely charged subfractions of the glycoprotein, i.e. the higher and lower sialylated ones are not present in the final product.

Another drawback may be found in the fact that when columns with immobilised antibodies are used to purify proteins by means of affinity adsorption, the acid eluting buffer induces sialic acid loss from the purified glycoprotein.

Although correlations between IgG glycosylation and lectin binding have been found, techniques using purification of the glycoprotein are considered unsuitable for application in the clinical practice.

Thus, the second glycoprotein i.e. haptoglobin was analysed in a system making use of the unique affinity of haptoglobin for hemoglobin. This approach, although successful for haptoglobin, will have very few equals as most plasma glycoproteins do not bind to another non-glycosylated protein which is readily available.

Still, the results obtained with haptoglobin merit further clinical trials. For instance, it would be of interest to follow cancer patients during periods of remission and relapse of their disease. A fundamental question to be answered concerns the mechanism behind changes in glycosylation of proteins produced by the liver, due to the presence of a tumor elsewhere in the body. Next, the study of haptoglobin glycosylation in other diseases such as RA (where the lectin MPA strongly binds haptoglobin) is a necessity, as well as in other autoimmune diseases such as SLE. Even within the RA class, subgroups such as RF seronegatives and vasculitis patients are of interest. These and other clinical studies remain to be initiated.

As the third application of the technique a lectin-enzyme immunoassay for HCG was developed. In comparison with IgG and haptoglobin, more is known about its carbohydrate chains and their modifications in relation to hydatidiform mole and choriocarcinoma. Unexpectedly, the lectin-enzyme immunoassay was able to detect important differences between the HCG samples used. Unexpectedly, because most lectins showed a high affinity for the monoclonal anti-HCG used. However, thanks to a phenomenon yet to be explained, the HCG binding to the antibody decreased the unwanted direct binding of the lectin to the antibody. Otherwise the discriminating power of the assay would have been decreased by the high blank binding. A second phenomenon which remains

unexplained is the lower RCA and PNA binding to HCG from hydatidiform mole or choriocarcinoma patients. Others have claimed that in these patients RCA and PNA reactive HCG is increased. In any case, the application of lectin-enzyme immunoassays is feasable even without deglycosylation of the antibodies, as the results with HCG show.

For a number of applications a set of lectin-enzyme binding assays should be used with a certain panel of lectins. For instance, a glycoprotein having an increased fucose content and produced by one tumor could be different from the same glycoprotein having an increased terminal galactose content and produced by a different tumor. Our results obtained with haptoglobin and with HCG suggest that with a certain set of lectins one may be able to detect several different changes in glycosylation.

The comparison between the results obtained with the lectin-enzyme binding assays and other lectin techniques should be made with caution, as differences in the binding properties may exist between immobilized lectins and lectins in solution (Montreuil et al., 1983). The same holds true for comparisons with crossed affino-immunoelectrophoresis (Salier et al., 1980). Not always will changes in the glycosylation lead to changes in lectin binding, even when the affinity of the lectins for the glycans have changed. It could be imagined, for instance, that α1-6 fucosylation of a biantennary glycan will lead to an increase of LCA binding. However, if, at the same time, the branching pattern of the glycan changes from biantennary to triantennary or tetraantennary, this will lead to a decrease in LCA binding. Thus, the simultaneous change of a glycan's structure towards increased affinity as well as towards decreased affinity will lead to an unchanged binding of the lectin used, although the glycan in question is different from its original structure in two respects. Besides lectin-enzyme immunoassays, other lectin based (or monoclonal anti-carbohydrate antibody based) techniques suffer from this same phenomenon.

Changes in the glycan structure may occur without a change in affinity of a certain lectin for the glycan. In such a case the lectin in question is unsuitable to detect this modification. At present, the selection of lectins on a trial and error basis is probably the fastest way to find meaningful combinations of the triad glycoprotein, disease and lectin.

Applications of lectin-enzyme binding assays outside the field of medicine may also be thought of. For instance, the study of the specificity of glycosyltransferases and glycosidases towards neoglycoconjugates or other glycoproteins immobilized in microtiter plate wells is possible after incubation of the enzyme, followed by determination of changes in lectin-binding.

The study of the glycosylation of glycoproteins produced by recombinant techniques for therapeutic use, such as clotting factors, will be easy using lectin-enzyme immunoassays. In such proteins the right glycosylation is of paramount importance to ensure maximum stability and to avoid uptake by membrane lectins due to incomplete glycosylation. One could imagine the selection of the right clones on the basis of the glycoprotein reactivity in lectin-enzyme immunoassays.

The techniques described in chapter 4 allow a detailed description of the lectin binding specificity. These techniques will gain importance as more BSA-glycoconjugates, more purified plasma glycoproteins and more lectins become available.

In short, the work described here has lead to the development of the concept of lectin-enzyme binding assays, in particular the lectin-enzyme immunoassays; new tools for the analysis of protein glycosylation.

SUMMARY

This work describes the development and application of lectin-enzyme binding assays, new analytical techniques, to investigate the glycosylation of glycoproteins. Glycoproteins are characterized by the presence of one or more covalently bound carbohydrate chains linked to a protein backbone. In human plasma the glycosylation of glycoproteins is subject to dynamic modifications, as is described in chapter 1, independent of the concentration of the glycoprotein. We studied the feasability of the use of lectins, specific carbohydrate binding proteins, in solid-phase enzyme-assays employing the lectins as immobilized binder (type I assay) or as soluble enzyme-bound binder (type II assay). A theoretical discussion of the use of lectins in ELISA techniques is given in chapter 1.

The potentials of the type I assay with an immobilised lectin are investigated indirectly. Chapter 2 describes experiments in which normal human serum with or without neuraminidase treatment was fractionated on a number of galactose-specific lectins bound to Sepharose. The bound and the unbound fractions were subsequently analysed by immunoelectrophoresis and by laser nephelometry. It is concluded that immobilized lectins will have very few applications in solid-phase enzyme-immunoassays using an antibody-enzyme conjugate to quantitate glycoproteins with terminal galactose groups, as a consequence of the phenomenon of cross-reactivity of many glycoproteins for the lectins used.

To develop the type II assay with immobilized antibodies and using a lectin-enzyme conjugate well-defined transferrin sialovariants were used, whose preparation and separation are described in chapter 3.

With these five transferrin sialovariants a lectin-enzyme immunoassay was developed with the lectin from Ricinus communis (RCA) as the galactose-specific lectin and peroxidase as the marker enzyme. In the final protocol both lectin and peroxidase are biotinylated and linked together by virtue of the biotin-binding protein Streptavidin. A number of variables have been investigated, resulting in an assay which shows a linear response with the number of terminal galactose groups on transferrin.

The lectin-enzyme binding assay may also be used to determine the lectin specificity by inhibition of the binding by simple saccharides. The principles of this lectin-enzyme binding inhibition assay, investigated with RCA as the lectin, are described in chapter 4.2.

With the lectin-enzyme binding assay the best results are obtained using biotinylated lectin and streptavidin-enzyme complexes or conjugates. However, the optimal ratio of the two compounds, leaving neither one in excess, and the most suitable dilution of the complex thus formed are not easily found, as may be concluded from the experiments described in chapter 3. In chapter 4.3 a new method is described which allows a fast and easy determination of the

point of equivalence of lectin-biotin and streptavidin-enzyme, employing avidin and biotinylated albumin coated microtiter plates. Another advantage of this method is that the lectin binding function is not used, making the method independent of lectin specificity and applicable for other binding proteins as well, such as antibodies, Protein A and others.

After establishment of the optimal composition and dilution of a large number of lectin-streptavidin-peroxidase complexes, the binding of each complex to a number of neoglycoproteins adsorbed in microtiter plates was studied (chapter 4.4). These BSA-glycoconjugates produced in vitro have extended glycan structures. Their lectin-enzyme binding gives more detailed information with respect to the lectin specificity as compared to the inhibition results described in chapter 4.2. In fact, both methods can be considered to be complementary to each other. New insights may be obtained in terms of the fine specificity of the lectin when studying the lectin-enzyme binding to neoglycoproteins, as examplified for the RCA lectin.

The concept of lectin-enzyme binding assay has also been used to determine the lectin binding to normal human plasma glycoproteins coated to microtiter plates. These experiments are described in chapter 4.5. It may be concluded that this technique compares favourably with affinodiffusion in gels or with lectin-column chromatography.

Three aplications of the developed assay were investigated with patient samples. First, IgG was purified from serum from blood donors, pregnant females and patients with rheumatoid arthritis. The IgG preparations were analysed with respect to their carbohydrate composition, and they were coated to microtiter plates. A correlation was found between the diminished galactose content of IgG from RA patients and the binding of a galactose-specific lectin (chapter 5.2). A possible explanation is put forward for the temporary remission of RA during pregnancy owing to the increase in IgG galactose content during pregnancy.

Second, the glycosylation of haptoglobin was investigated by lectin-enzyme binding after incubation of serum in hemoglobin-coated microtiter plate wells. Associations with certain diseases were noted. Haptoglobin in serum of a number of cancer patients showed elevated binding of some of the lectins used. The experiments are described and discussed in chapter 5.3.

Third, the HCG glycosylation was studied in serum from patients with HCG producing tumors and molar pregnancies, as well as from normal pregnant women. Malignancy-associated differences in HCG glycosylation were detected by the lectin-enzyme immunoassay in microtiter plate wells coated with monoclonal anti-HCG (chapter 5.4).

A general discussion on the results obtained and on the prospective areas of application of lectin-enzyme binding assays is given in chapter 6.

SAMENVATTING

Dit proefschrift beschrijft de ontwikkeling van lectine-enzym bindingsanalyses, een nieuwe techniek om de glycosylering van glycoproteinen te onderzoeken.

Glycoproteinen onderscheiden zich door de aanwezigheid van één of meer covalent gebonden koolhydraatketens. In het plasma van de mens is de glycosylering van de glycoproteinen dynamisch van aard, zoals in hoofdstuk 1 wordt beschreven, en onafhankelijk van de concentratie van het glycoproteine.

De toepasbaarheid van lectines, specifieke koolhydraat-bindende eiwitten, als geimmobiliseerde binders of als enzym-gebonden binders in ELISA technieken werd bestudeerd. Een theoretische discussie van het gebruik van lectines in ELISA technieken wordt gegeven in hoofdstuk 1.4. De mogelijkheden van type I bepalingen, waarbij het lectine geimmobiliseerd is, werden indirect onderzocht. Hoofdstuk 2 beschrijft experimenten waarin humaan serum, al dan niet behandeld met neuraminidase, gescheiden werd op een aantal Sepharose kolommen waaraan galactose specifieke lectines zijn geimmobiliseerd. De gebonden en de ongebonden fracties werden vervolgens geanalyseerd door middel van immunoelectroforese en laser nefelometrie. De conclusie wordt getrokken dat voor de combinaties van geimmobiliseerde galactose-specifieke lectines en antilichaam-enzym conjugaten weinig toepassingen zullen zijn om de eindstandige galactose groepen van glycoproteinen te kwantificeren, door het optreden van kruisreakties van vele glycoproteinen met de gebruikte lectines.

Voor het ontwikkelen van de type II bepaling met geimmobiliseerde antilichamen en lectine-enzym conjugaten maakten we gebruik van gedefinieerde transferrine sialovarianten, waarvan de bereiding en scheiding beschreven is in hoofdstuk 3.

Met deze vijf transferrine sialovarianten werd een lectine-enzym immunoassay ontwikkeld met RCA als de galactose-specifieke lectine en peroxidasé als het indicator enzym. In het uiteindelijke protocol werd een complex gebruikt van gebiotinyleerde lectine gekoppeld aan gebiotinyleerd peroxidase via streptavidine. Een aantal variabelen werd onderzocht. Het resultaat is een bepaling waarbij een lineair verband wordt gevonden met het aantal eindstandige galactose groepen van transferrine.

De lectine-enzym bindingsanalyse kan ook worden gebruikt om de specificiteit van lectines te bepalen in inhibitie experimenten met simpele suikers. Deze techniek wordt beschreven in hoofdstuk 4.2 voor het lectine RCA.

De beste resultaten van lectine-enzym bindingsanalyses worden verkregen door gebruik te maken van gebiotinyleerde lectines en streptavidine-enzym complexen of conjugaten. Echter, de optimale verhouding van deze constituenten, waarbij geen van beide in overmaat aanwezig is, was niet eenvoudig te bepalen, zoals de experimenten beschreven in hoofdstuk 3 aangeven. In hoofdstuk 4.3

wordt een nieuwe techniek geintroduceerd waarmee snel en eenvoudig het equivalentiepunt van gebiotinyleerde lectine en streptavidine-peroxidase wordt bepaald in microtiter platen gecoat met gebiotinyleerd albumine en met avidine. Een bijkomend voordeel van de ontwikkelde methode is dat geen gebruik gemaakt wordt van de lectine bindingsfunctie, waardoor zij onafhankelijk is van de specificiteit van het lectine en algemeen toepasbaar voor andere bindende eiwitten zoals antilichamen, Protein A, etc.

Na het vaststellen van de optimale verhouding en verdunning van een aantal lectine-streptavidine-peroxidase complexen werd vervolgens de binding bestudeerd aan een aantal neoglycoproteinen geadsorbeerd in microtiter platen. Deze in-vitro geproduceerde BSA-glycoconjugaten kunnen langere suikerstructuren bezitten. De binding van lectine-enzym complexen, zoals beschreven in hoofdstuk 4.4, geeft meer gedetailleerde informatie over de lectine specificiteit als de inhibitie techniek, hoewel beide technieken als complementair kunnen worden gezien. De binding van het door ons gebruikte voorbeeld van het RCA lectine leverde nieuwe gegevens op over details van de specificiteit van het lectine.

Het idee van de lectine-enzym bindingsanalyse kan ook worden gebruikt om de binding van lectines aan normale plasma-eiwitten te bepalen. Deze experimenten met aan microtiter platen gecoate plasma-eiwitten staan beschreven in hoofdstuk 4.5. De techniek biedt een aantal voordelen vergeleken met affinodiffusie of lectine-kolom chromatografische technieken.

Drie toepassingen van de ontwikkelde bepalingstechniek werden onderzocht met patientenmateriaal. Allereerst werd IgG gezuiverd uit bloed van RA patienten, zwangere vrouwen en bloeddonoren, en de IgG koolhydraatsamenstelling geanalyseerd. Ook werden de IgG preparaten gecoat aan microtiter platen en werd vervolgens de binding van lectines bestudeerd. Er werd een correlatie gevonden tussen het verminderde galactose gehalte van IgG van RA-patienten en de binding van een galactose-specifieke lectine (hoofdstuk 5.2). Een mogelijke verklaring wordt geopperd voor de tijdelijk remissie van RA tijdens de zwangerschap, gezien de stijging van galactose in IgG tijdens deze periode.

Als tweede werd de glycosylering van haptoglobine onderzocht met de lectine-enzym bindingsanalyse, na incubatie van serum in hemoglobine-gecoate microtiter plaatjes. Associaties met zekere ziekten werden gevonden. Haptoglobine van een aantal kanker-patienten vertoonde verhoogde lectine-binding met enkele van de gebruikte lectines. Deze experimenten staan beschreven in hoofdstuk 5.3.

Als derde werd de HCG glycosylering bestudeerd in serum van patienten met HCG producerende tumoren, mola zwangerschappen en gezonde zwangere vrouwen. De lectine-enzym immunoassay, gebruikmakend van microtiter platen gecoat met monoclonaal anti-HCG, toonde verschillen aan in HCG glycosylering gerelateerd aan de maligniteit (hoofdstuk 5.4).

Een algemene discussie van de verkregen resultaten en van de mogelijke toepassingsbieden van lectine-enzym bindingsanalyses wordt gegeven in hoofdstuk 6.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 5 juni 1946 te Eindhoven. Na het behalen van het HBS-B diploma in Delft werd aangevangen met de studie Scheikunde aan de Universiteit van Groningen. Na tussentijds de militaire dienstplicht te hebben vervuld als officier der artillerie werd de studie voortgezet aan de Universiteit van Leiden. In 1974 werd het doctoraalexamen behaald met twee hoofdvakken: Klinische Chemie (Prof. Dr. A.A.H. Kassenaar) en Organische Chemie (Prof. Dr. E. Havinga †), en met als bijvak Radiochemie.

Na een verblijf in de Verenigde Staten werd in 1976 aangevangen met de opleiding tot klinisch chemicus bij het Centraal Klinisch Chemisch Laboratorium van het Academisch Ziekenhuis Dijkzigt te Rotterdam (hoofd: Prof. Dr. B. Leijnse), welke opleiding in 1980 resulteerde in de erkenning tot klinisch chemicus. Van 1980 tot 1986 werd de functie vervuld van klinisch chemicus aan het Klinisch Chemisch Laboratorium van de Dr. Daniël den Hoed Kliniek te Rotterdam (hoofd: Dr. W.G. Haije). In 1986 volgde de aanstelling tot klinisch chemicus bij de sektor Klinische Chemie en Hematologie en in 1988 als hoofd van de afdeling Klinisch Chemisch Laboratorium van het Diagnostisch Centrum SSDZ te Delft.

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