PREPARATION AND APPLICATION OF LECTIN-GOLD COMPLEXES

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SUMMARY: The results obtained with various plant and animal tissues as well as pathogenic cells demonstrate that lectin-gold complexes enable localization of sugar residues at the ultra structural level. Provided that sugar residues are not altered during processing, the lectin-gold method represents undoubtedly a simple and powerful approach for localizing glyco-conjugates. Because of the electron dense gold marker, the labeling obtained is always of high resolution and a good correlation of markers and the cellular compartments can be easily and repeatedly established. In our studies, incubation of thin sections of the human conjunctival goblet cells having N. acetyl D-Galactoseamine rich mucus droplets with the HPA-GC revealed numerous gold particles in the electron micrographs.

Key words: Lectin-gold complexes, gluco-conjugates, post-embedding.

INTRODUCTION

Although known for nearly a century, it is only during the last two decades that lectins have been recognized as a class of proteins or glycoproteins, having binding sites for carbohydrates. In view of the increasing evidence that carbohydrates are strongly involved in many biological mechanisms, such as cell signaling, cell attachment, cell adhesion, and self or non-self recognition, it is not surprising that lectins have become the focus of intense interest not only in immunology (35) and biochemistry (9,11,29,35), but also in cell biology (22,42).

There are many reasons for the current interest in lectins. Prominent among these is their usefulness in detecting and studying carbohydrates in solution and on cell surface. Lectins are usually described in terms of mono-saccharine specificity. However, they display also the ability to recognize fine differences in more complex structures, as recently illustrated by Debray *et. al.* (14) in a detailed analysis of the specificity of 12 lectins. This means that lectins do not recognize only sugar residues in a terminal non-reducing position of polysaccharides, but also bind to carbohydrate moieties of glycoproteins. Another reason is that lectins are thought to act as recognition determinants in a variety of biological systems in plants and animals, including host/pathogen interactions and host resistance to infections (7,53).

Recent developments in cell biology and biochemistry have disclosed that lectins are not only powerful tools for extra-cellular localization of carbohydrate containing molecules, but are also useful probes for the localization and distribution of a given carbohydrate within the cell compartments (48).

Since lectins are not electron-opaque and free of enzymatic activity, lectin binding sites cannot be directly visualized at the electron microscope level. A variety of techniques dealing with the conjugation of lectins with markers, including enzymes (e.g. peroxidase), electron-opaque molecules (e.g. ferritin) or

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metal particles (e.g. colloidal gold), have been proposed to circumvent this problem. Once tagged to a specific marker, lectins can be used in a variety of ways for the ultra-structural localization of carbohydrate residues. They can be applied in a pre-embedding approach prior to or after tissue fixation (30) or in a post-embedding approach after tissue sectioning (6,48). The latter approach includes a direct method, where the dense marker is complexed to the lectin, and an indirect two-step method, where the marker is linked to a glycoprotein that displays a strong affinity for the lectin used (6). Pre and post-embedding approaches are useful in transmission electron microscopy and also in scanning electron microscopy, where distribution of carbohydrates over different areas of the cell surface can be easily observed, provided that appropriate marker are used (15,39).

DEFINITION AND PROPERTIES OF LECTINS

Since the introduction of the term 'lectin' (from the latin lego/lectum, meaning I pick out or choose) by Boyd and Shapleigh (10) to describe a group of proteins or glycoproteins of plant origin, that agglutinate cells, the subsequent discovery of similar substances in both animal and plant tissues has prompted the need to reach a common agreement for defining a lectin. Goldstein *et. al.* (23) have proposed to define a lectin simply as "a carbohydrate-binding protein or glycoprotein of nonimmune origin which agglutinates cells and/or precipitates glyco-conjugates". The Nomenclature Committee of the International Union of Biochemistry (17) recommended the adoption of this definition with, however, some minor modifications such as deletion of the term glycoprotein.

This definition implies that lectins are multivalent or, in other words, that they posses at least two sugar-binding sites that enable them to agglutinate animal or plant cells bearing surface glyco-conjugates. The emphasis on 'nonimmune origin' is of major importance in the definition, since it allows us to distinguish lectins from anti-carbohydrate antibodies, which exhibit also the ability to agglutinate cells. Since most lectins are isolated from plants that do not synthesize immunoglobulins and, furthermore, unlike antibodies, are known to vary greatly in molecular size, amino acid and conformational structure, their nonimmune origin can be claimed without ambiguity.

CARBOHYDRATE - BINDING SPECIFICITY OF LECTINS

The determination of the carbohydrate-binding specificity of a lectin is of major importance for subsequent biochemical, immunochemical, and cytochemical applications. Various procedures have been developed to establish the affinity of a lectin for a specific sugar. Among these, hemagglutination reaction (45,52), direct ultraviolet spectroscopy (8,28), fluorescence spectroscopy and elution from solid-phase adsorbents (12), and precipitation studies (46) have been most often applied and have, undoubtedly, been highly informative. Recently, the chromatography of glycoprotein structure through lectin columns has also provided valuable information regarding the carbohydrate-binding specificity of lectins (13,14).

With the expansion of our knowledge sugar-binding sites of lectins, it became apparent that lectins could be divided into several broad categories according to their specificity. A compilation of the well-established carbohydrate-binding specificities for a number of lectins presented in Table 1.

Except for a few exceptions, lectins interact usually with the non-reducing, terminal glycosyl groups of polysaccharides and glycoproteins. Among the exceptions, concanavalin A and wheat germ agglutinin are worthy of mention. Thus, in addition to interacting with α-glucopyranolsyl terminal groups, concanavalin A binds internal α-mannopyranosyl residues (24). Similarly, the wheat germ agglutinin is able to interact with internal N-acetyl glucosamine residues (1).

Variations of at C-2 of sugars are not involved in lectin binding. As an example, concanavalin A, which binds preferentially mannose, reacts also, but to a lesser extent, with glucose. In contrast, the C-4 hydroxyl group of carbohydrates seem strongly involved in lectin-binding specificity. Allen *et. al.* (1) have demonstrated that N-acetylglucosamine-binding lectins do not exhibit affinity for N-acetylgalactosamine, and vice versa. Similarly, mannose/glucose lectins do not interact with galactose (2).

The simple sugar that inhibits lectin binding may not

Table 1: Carbohydrate-binding specificities of some commonly used lectins.

Lectin	Source	Nominal speficity a	Molecular weight
N-Acetylglucosamine group			
Wheat germ aggluthin (WGA)	Triticum vulgaris (embryos)	GlcNAc>Neu-5-Ac	36.000
Solanum Tuberosum agglutingn (STA)	Solanum tuberosum (tubers)	D-Glc-Nac	50.000
Daturda stramonium agglutining (DSA)	Datura stramonium (thorn apple)	Glc-Nac=Gal-β1, GlcNac	86.000
Lycopersicon esculentum agglutinin (LEA)	Lycopersiconðesculentum (tomato)	GlcNAc	71.000
N-Acetylgalactsamine group			
Helix pomatia agglutinin	Helix pomatia (Roman snail)	GalcNAc-α1, 3GalNac>αGalNAc	79.000
Glycine max agglutinin (SBA)	Glycin max (sobyean seeds)	D-GalNAc	110.000
Banderiraea simplicifolia agglutinin (BS-1A4)	Bandeiraea or Griffonia Simplicifolia (seeds)	α-D-GalNAc	114.000
Vicia villosa agglutinin A4 (VVA-A4)	Vicia villosa (hairy vetch)	GalNAc-α-1,3-Gal=α-GalNAcα-GalNAc	139.000
Vicia villosa agglutinin B4 (VVA-B4)	Vicia villosa	α-GalNAc	134.000
Dolichos biflorus agglutinin (DBA)	Dolichos biflorus (horse gram)	GalNAc-α-1,3 GalNAc>>α-GalNAc	140.000
Glucose/mannose group			
Concenavalin A (Con A)	Canavalia ensiformis (seeds)	α-Man>α-Glc>GlcNAc	102.000
Vicia faba agglutinin (VFA)	Vicia faba (seeds	α-Man>α-Glc>GlcNAc	50.000
Pisum sativum agglutinin (PSA)	Pisum sativum (seeds)	α-Man>α-Glc=GlcNAc	49.000
Lens culinaris agglutinin (ICA)	Lens culinaris	α-Man>α-Glc>GlcNAc	49.000
Galactose group			
Ricinus communis agglutinin (RCA)	Ricinus communis	β-Gal>α-Gal>>GalNAc	120.000
Ricinus communis toxin	Ricinus cummunis	β-and α-Gal>GalNAc	60.000
Abrus preatorius agglutinin (APA)	Abrus precatorius	β-Gal>GalNAc	134.000
Viscum Album agglutinin (VAA)	Viscum album (European mistletoe)	β-Gal	115.000
Euonymus europaeus agglutinin (EEA)	Euonymus europaeus (Spindle Tree)	α-D-Gal-1,3-D-Gal>L-Fuc-1,2-Gal	166.000
Allo A lectin Jacalin	Allomyrina dichotoma	β-Gal>α-Gal	65.000
	Artocorpus integrifolia	β-Gal>α-Gal	39.500
L-Fucose group			
Lotus tetragonolobus agglutinin (LTA)	Lotus tetragonolobus	α-L-Fuc>L-Fucα1,2Galβ-1,4-GlcNAc>>L-Fuc-1,2 Gal-β-1,3-GlcNAc	170.000
Ulex eupopaeus agglutinin 1 (UEAI)	Ulex europeaus (gorse)	α-L-Fuc	170.000
Anguilla anguilla agglutinin	Anguilla anguilia (fresh water eel)	α-L-Fuc	40.000
Sialic acid group		α-Neu-5-Ac>α-Neu-5	
Limax flavus agglutinin (LFA)	Limax flavus limulus		
Limulus polyphemus agglutinin (LPA)	polyphemus (horseshoe crab)	Neu-5-Ac-α-2,6 GalNAc>Neu-5-Ac	400.000

a Abbreviations : GlcNAc-N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Man, mannose; Gl, glucose; Gal, galactose; L-Fuc, L-fucose; Neu-5-Ac N-acetylneuraminic acid or sialic acid>, Aromatic effect results in incrising bindings.

be exactly the same as the sugar group to which the lectin binds in the tissues (36). In such instances, two lectins with similar simple sugar specificities may not bind necessarily to identical tissue components. Therefore, histo- and cytochemical observations should always be carefully interpreted with respect to the preferential sugar group recognized by the lectin under study.

NATURE OF CARBOHYDRATE - LECTIN INTERACTIONS

Although little is known as to what kind of forces may be involved in carbohydrate-lectin interactions, there is some evidence that polar interactions, such as hydrogen bonds, and dipole interactions play an important role in the formation of lectin-sugar complexes (5). Polar interactions between hydroxyl groups of carbohydrates and the polar side chains of amino acid residues within a lectin's hydrophilic binding site can be provided an ideal model for carbohydrate-lectin interactions (25).

Factors such as pH, temperature, and ionic strength are closely implicated in the formation of carbohydrate-lectin complexes (40). A slight modification of such parameters may induce disruption of the complexes. Monsigny *et. al.* (41) reported that the specificity of lectins may also be altered by chemical modification. These authors observed a considerable decrease in lectin-binding sites when wheat germ agglutinin was succinylated. They suggested that the isoelectric point of this lectin changed upon succinylation.

LECTINS AS TOOLS IN CYTOCHEMISTRY

Because of their carbohydrate-binding specificity, lectins have proved to be powerful tools for the investigation of the cell surface architecture (37,43). Papers dealing with the interaction of various lectins with plant, animal, and microorganism cells started to appear with increasing frequency after the first demonstration that concanavalin A could be used as a cytochemical reagent for studying the cell surface coat of mammalian cells (4, 56). The rationale for these studies is that they could not only allow the mapping of the cell surface carbohydrates, but also contribute to a better understanding of various surface related biological phenomena, such as cell-cell interactions, immune response, morphogenesis drug resistance, or cell attachment.

Since they are not electron-opaque, lectins cannot be directly visualized at the electron microscope level, and consequently have to be conjugated to an electron-dense marker (31). Several possibilities exist for the type of marker that can be used. Among the enzymatic markers, horseradish peroxidase (HRP) has been the most widely applied. HRP once bound to lectins, is easily detectable via an osmiophilic raction using 3,3'-diaminobenzidine as the substrate (3). However, this technique has a limitation since diffusion of the reaction products away from the original binding sites does not allow an accurate localization of a given carbohydrate. Lectin-binding sites can also be visualized by a variety of particulate markers. These include ferritin (44), hemocyanin (54), iron-dextran (38), and iron-mannan (49). More recently, colloidal gold has been introduced in the field of cytochemistry as an alternative to previous particulate markers (32,50). Because gold particles are electron dense, uniform in size, and can be reproducibly and easily prepared, they represent actually most attractive markers for both scanning and transmission electron microscopy (16).

In contrast to the extensive use of lectin-gold complexes in the cell surface studies (30), their application for the detection of intracellular receptors is relatively new (48). Since early 1980s, an increasing number of papers dealing with post-embedding sugar localization by means of gold-complexed lectins have appeared, thus demonstrating the usefulness of such probes in a variety of biological systems.

PREPARATION OF LECTIN-GOLD COMPLEXES

Colloidal gold

Of the particulate marker, colloidal gold has generated increasing interest as an electron-opaque, noncytotoxic, and stable cytoplasmic marker that can be observed by various modes of microscopy (26). Colloidal gold was first used in 1962 for studies of intracellular exchange in amoeba (Chaos chaos) (19), but its first application as a specific marker for transmission electron microscopy was reported by Faulk and Taylor (18). It was then introduced as a marker for scanning electron microscopy in 1975 (33) and for fluorescence microscopy (34). Roth and Wagner (51) were the first

to demonstrate that lectin-gold complexes were valuable reagents not only for cell surface labeling, but also for studies on internalization. Since then, a whole variety of ligands, such as immunoglobulins, lectins, enzymes, toxins, protein A and avidin, have been successfully conjugated to colloidal gold and applied in pre- and post-embedding labeling of numerous animal and plant tissues (47,48).

Colloidal gold is a negatively charged hydrophobic sol whose stability is maintained by electrostatic repulsion (57). When electrolytes are added to colloidal gold, cohesion of the particles occurs and results in flocculation. However, such particle aggregation can be prevented by prior addition of proteins, provided that appropriate conditions, including ionic concentration, are provided.

The general principles underling the preparation of colloidal gold have been established in previous excellent reviews (27,48). Gold colloids are prepared from tetrachloroauric acid ($HAuCl_4$), which must be reduced by an appropriate agent (30). The most common used reducing agents are phosphorus, formaldehyde, ethanol, tannic acid, ascorbic acid, and sodium citrate. Depending on the method used, gold particles can be prepared in a size ranging from 3 to 150 nm. However, for transmission electron microscope purposes, sizes ranging from 5 to 20 nm are the most suitable.

Preparation of 14 nm gold particles

Colloidal gold with particles averaging 14-16 nm in diameter can be prepared according to the method developed by Frens (20). Five milliliters of 0.2% tetrachloroauric acid is added to 95 ml of double, distilled water and the solution is allowed to boil for 5 min. Four milliliters of 1% sodium citrate is then added as quickly as possible. Upon gentle boiling, the solution turns blue, becomes purple, dark red, and finally orange-red. The reduction process is generally achieved after 10-15 min of boiling.

Adsorption of lectin to colloidal gold

General Considerations about Adsorption of Proteins to Colloidal Gold:

Under appropriate conditions, colloidal gold binds proteins by noncovalant electrostatic adsorption, which tends to stabilize the sol to flocculation. The stability of gold-protein interaction is not completely understood, although it is well established that various physicochemical factors influence the adsorption process. In this regard, the study of Geoghegan and Ackerman (21) has demonstrated that adsorption of proteins to colloidal gold is not only pH-dependent but also closely related to the concentration and the molecular weight of the proteins as well as to ionic strength.

As a general rule, maximal adsorption of proteins to gold occurs at pH values close to or slightly basic to the isoelectric point of a given protein (21). Thus depending on the isoelectric point of a protein, the pH of the gold solution must be adjust either by addition of 0.2 MK_2CO_3 to raise it or by addition of 0.1 NHCl to lower it. In their fundamental investigations on the conditions for protein adsorption, Geoghegan and Ackerman (21) pointed out that colloidal gold may plug the pores of the pH electrode. They suggested that the addition of a few drops of 1% aqueous polyethylene glycol (PEG) 20.000 to an aliquot of colloidal gold could stabilize it before the insertion of the electrode. This procedure is now currently used in most laboratories where cytochemical experiments are conducted.

In order to provide optimal conditions for complex formation, the ionic concentration of protein solutions must be very low. Geoghegan and Ackerman (21) determined that concentrations of NaCl as low as 0.1 M prevented the formation of a stable complex. They concluded that proteins should be dissolved in very dilute salt solutions or distilled water if possible prior to complex formation.

Under optimal conditions of pH and ionic concentration, the amount of protein needed for stabilizing the gold solution against flocculation has to be established (48). An easy and reliable procedure, developed by Horisberger *et. al.* (33), consists in exposing a constant amount of colloidal gold (5 ml) to decreasing amount of proteins (1 ml). After 5 min, 1 ml of 10 % NaCl solution is added. Flocculation is either judged visually through a color change from red to violet and finally to light blue or estimated spectrophotometrically at 525-540 nm. Usually, the minimal amount of protein that prevents a color change from red to blue in considered to be stabilizing. However, for the preparation of the complex,

Table 2: Optimal conditions for the preparation of lectin-gold complexes.

Lectin	Sugar specificity	pH of colloidal gold	Amount of Lectin needed to stabilize 10 ml colloidal gold (μg)	Optimal pH of labeling
Concanavalin A	α-D-Mannose	8.0	250	8.0
Dolichos biflorus agglutinin	α-D-Glucose N-Acetyl-D			
Glycine max	galactosamine	6.0	240	7.2
agglutinin	N-Acetyl-D	6.1	65	7.4
Helix pomatia agglutinin	galactosamine N-Acetyl-D-	7.4	100	7.4
Lens clunaris agglutinin	galactosamine α-Mannose fucosyl	6.9	200	7.4
galactosamine Lotus tetragonolobus	groups			
agglutinin	α-L-Fucose	7.0	200	7.4
Ricinus communis agglutinin I	α-(1-4)-Galactose	8.0	60	8.0
Ulex europaeus agglutinin	α-L-Fucose	6.3	250	7.4
Viscum album agglutinin	β-(1-4)-D-Galactose	9.0	200	7.4

most authors use a 10-fold excess of the stabilizing agent.

Formation of lectin - gold complexes

Lectins were among the first macromolecules to be conjugated to colloidal gold (32,33). However, the failure of these earlier scientists to prepare stable and reproducible lectin-gold complexes can be explained by the fact that optimal conditions for complex formation were not considered in their own right. With the increase of knowledge concerning the physiochemical properties of lectins and the basic parameters for protein-gold interaction, the preparation of lectin-gold complexes has become an easily reproducible process.

Table 2 shows the optimal conditions for direct complex formation of selected lectins with colloidal gold. The isoelectric point of each lectin and minimal amount of protein needed for stabilization are presented.

Conditions for direct complex formation

Most lectins can be directly conjugated to colloidal gold, provided that optimal conditions for complex formation are well determined. For preparation of lectin-gold complexes, colloidal gold with an average particle diameter of 8 or 14 nm is generally used. Highly purified lectins can be obtained either from Sigma Chemical Co (St. Louis, Mo) or Calbiochem-Behring Corp (La Jolla, Calif). For the preparation of lectin-gold complexes, the following protocol is recommended :

Figure 1: Direct and indirect lectin-gold labeling. In the direct technique, the lectin molecules tagged to colloidal gold will interact with their specific sugar present at the exposed surface of the tissue section. In the indirect technique, the unlabeled lectin will first react with its corresponding sugar and then a gold-labeled protein will interact with the lectin.

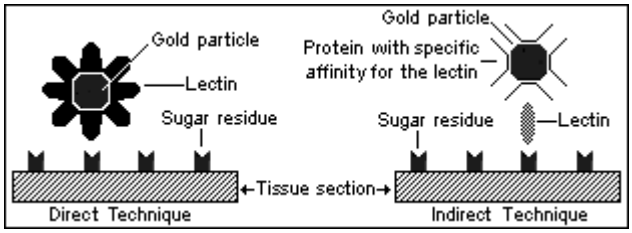
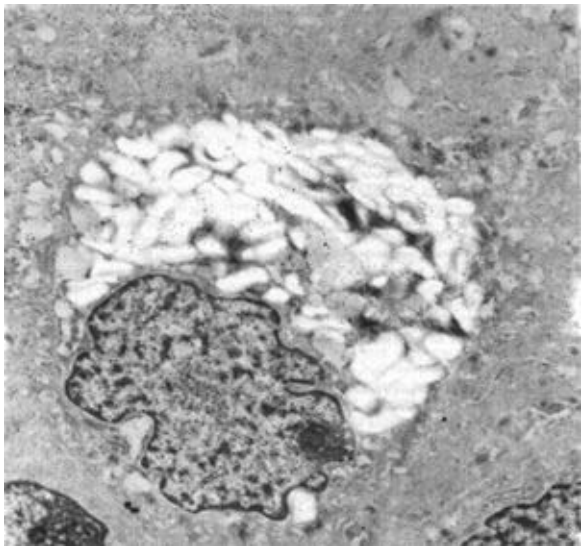


Figure 2: An electron micrograph of goblet cell of normal human conjunctiva fixed in glutaraldehyde, embedded in Agar Resin 100, and incubated with Helix pomatia lectin-gold complex (HPL-GC). Black gold particles with 14 nm representing Helix pomatia lectin-binding sites are seen over mucus droplets. x13500.



1. The pH of gold sol is carefully adjusted according to the isoelectric point of the lectin under study (Table 2).

2. The minimal amount of lectin needed to stabilize 10 ml of colloidal gold at the appropriate pH is determined (Table 2).

3. A 10-fold excess of the optimally stabilizing lectin amount is dissolved in 0.1 ml of double-distilled water and placed in a centrifuge tube.

4. Ten milliliters of colloidal gold (at the optimal pH for binding) is added as quickly as possible to the lectin solution.

5. Adsorption of the lectin on the surface of the gold particles occurs upon mixing. The mixture is further stabilized by the addition of 0.5 ml of 1% aqueous PEG 20.000.

6. The resulting complex is centrifuged to remove excess of unbound lectin. Centrifugation is performed at 4°C in a Sorvall or Beckman ultracentrifuge, for either 30 min (14 nm gold particles) or 45 min (8 nm gold particles) at 25.000 rpm using a Ti-50 rotor.

7. After the centrifugation step, three well-delineated phases are visible in the tube : a clear supernatant that contains free, unbound lectin, a dark zone that consist of unstabilized, aggregated gold particles, and a dark-red sediment in the bottom of the centrifuge tube. The colorless supernatant is aspirated as completely as possible and discarded. The dark-red sediment, which corresponds to the lectin-gold complex, is recovered in 0.5 ml of 0.1 M phosphate-buffered saline (PBS) containing 0.02% PEG 20.000. The pH of the PBS-PEG is brought to the optimal pH of labeling. This stock solution is placed in an Eppendrof tube and stored at 4°C. Note that all lectin-gold complexes have the property to retain their bioactivity for at least 6 months.

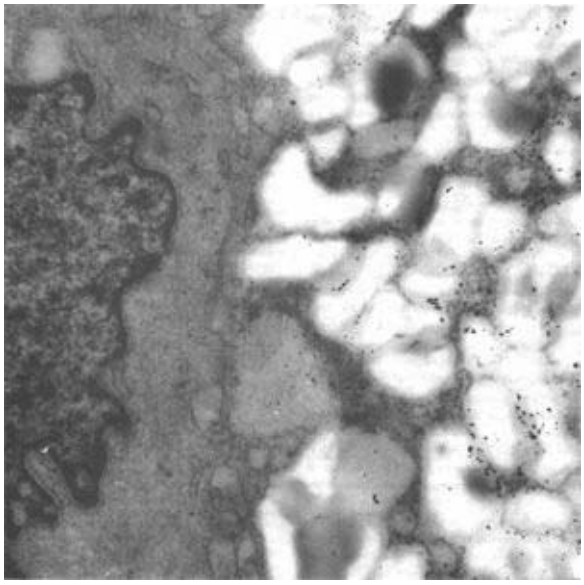
Cytochemical labeling

Both direct and indirect labeling methods can be applied to ultra-thin tissue sections for the localization of intracellular lectin receptors (6). As a general rule, incubation are performed at room temperature since the molecular properties of most lectins are known to vary depending on temperature (48). In order to avoid any desiccation, it is recommended to perform the experiments in a moist chamber.

Direct cytochemical labeling

The one-step, post-embedding method is depicted in Figure 1. The principle of this method is that, upon

Figure 3: Higher magnification of one of the cytoplasmic part of goblet cell. The colloidal gold particles are intensely observed over N-Acetyl D-Galactosamine rich mucus droplets. x 28500.



incubation of the tissue section with the lectin-gold complex, the lectin molecules adsorbed on the surface of the gold particle bind their corresponding carbohydrate molecules exposed at the surface of the tissue section. At the electron microscope level, the lectin-sugar interaction is easily visualized by the gold particle, due to its high electron density.

Indirect cytochemical labeling

The principle of two-step, post-embedding labeling is illustrated in Figure 1. Thin tissue sections are first incubated with the unlabeled lectin, which binds specifically to its corresponding sugar molecule. The sugar-lectin interaction is then detected through the use of a gold-complexed glycoprotein, the latter being chosen for its high affinity for the lectin under study.

Ultra structural localization of N-Acetyl D-Galactosamine residues

Detection of galactose amine residues was done by means of the Helix Pomatia Agglutinin-Gold Complex (HPA-GC) (6). The lectin was complexed to gold at pH 7.4. Grids were floated on a drop of PBS-PEG pH 7.4, then transferred onto a drop of the HPA-GC diluted 1:10 in PBS-PEG, pH 7.4.

Incubations of thin sections of the human conjunctival goblet cells having N-Acetyl D-Galactosamine rich mucus droplets, with the HPA-GC revealed that numerous gold particles were present over the mucus droplets (55) Figures 2 and 3.

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