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Invited Review

Carbohydrates and soluble lectins in the regulation of cell adhesion and proliferation

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Summary. There is a large body of suggestions that complex carbohydrates play a role in the regulation of cell adhesion and cell proliferation. Many reports have emphasized that proteoglycans, glycoproteins or glycolipids are participating to cell adhesion mechanisms. The use of polyvalent anti-carbohydrate antibodies and plant lectins as well as the use of glycosylation inhibitors suggested that cell proliferation can be modulated by surface carbohydrates. The dating experiment of Burger and Noonan (1970) showing restoration of contact inhibition of malignant cells by monovalent concanavalin A was a determining experiment. However, in the latter as in the others, no precise mechanism was demonstrated how carbohydrates can be involved in adhesion and proliferation. New insights were opened with the discovery of vertrebrate membrane-bound and soluble lectins. The latter generally display agglutinating activities in *in vitro* systems, suggesting that they were potential cell adhesion molecules, by forming bridges between cell surface carbohydrates. These polyvalent molecules may be also considered as clustering agents for their cell surface ligands, consequently generating signals for cell proliferation and/or differentiation.

Key words: Carbohydrates, Lectins, Regulator, Adhesion, Proliferation

Introduction

In the recent years, a considerable interest was directed to carbohydrate-binding proteins (lectins), since they have been found as essential molecules for homing of immune cells (Monsigny et al., 1983; McEver, 1991; Springer, 1991; Durand, 1992; Lasky and Rosen, 1992; Bevilacqua and Nelson, 1993; Stoolman, 1993). The membrane-bound, calcium-dependent lectins known as selectins are not the only carbohydrate-binding proteins found in mammals, but soluble lectins of different carbohydrate specificities have been isolated or detected in several tissues. Although their expression *in vivo* is generally developmentally regulated, they often accumulate intracellularly and are externalized at crucial period of the development, suggesting that they are playing a role in cell contacts. However, other types of molecules and other types of cell adhesion mechanisms have been described which do not postulate a role for the carbohydrate-binding proteins. Similarly, *in vitro* studies using cross-linking agents indicate that efficient signal transduction is dependent upon clustering of surface molecules, although the postulated endogenous effectors are not known. This review will try to summarize the major mechanisms involved in cell adhesion and in the surface regulation of cell proliferation, and to analyze the possible involvement of soluble lectins, based on recent data of the literature.

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The major cell adhesion processes

Various types of cell adhesion mechanisms have been described in the literature (summarized in Fig. 1) which, theoretically, are not mutually exclusive. They involve protein-protein homophilic interactions, lipid-lipid interactions, protein-protein heterophilic interactions, protein-carbohydrate heterophilic interactions, and carbohydrate bridging by soluble polyvalent proteins. Most of them can be strictly calcium-dependent or calcium-independent.

Protein-protein homophilic interactions

The one which has been studied extensively during the last twenty years is the mechanism of *homophilic* interactions (Edelman, 1986; Rutishauser et al., 1988; Salzer and Colman, 1989; Brackenbury, 1990; Chuong, 1990; Edelman and Crossin, 1991a,b; Filbin et al., 1990; Filbin and Tenekoon, 1991, 1993). It is supposed to take place by protein-protein interactions between peptide domains of the same molecule (or molecules with

extensive sequence homologies) termed as the homophilic domain. The model molecule for this type of interaction is the neural cell adhesion molecule, N-CAM. By extension a large number of molecules of the superfamily of immunoglobulins are considered as participating to cell adhesion with a similar mechanism. They include the L1/Ng-CAM antigen, the myelin-associated glycoprotein MAG (Schachner, 1989; Walsh, 1989), the myelin-oligodendrocyte glycoprotein MOG, the major glycoprotein of the peripheral nervous system P0, the carcinoembryonic antigen CEA, the non-specific carcinoembryonic antigen NCA as major members in vertebrates. In *homophilic* interactions, N-CAM on the one cell is interacting with N-CAM on the other cell (Fig. 1a). This could occur when the cells are identical (*homotypic* interaction) or when the two cells are different (*heterotypic* interaction). The demonstration that the polypeptide chain of these glycoproteins is actually involved in the adhesion is still lacking. In contrast, recent experiments of Murray and Jensen (1992) suggest that the homophilic nature of the adhesion mechanism involving N-CAM is not biologically proven, since the cell molecules interacting with N-CAM coated surfaces are unrelated with N-CAM. Recent data on the role of the carbohydrate moiety of these highly glycosylated glycoproteins are somewhat contradictory. HNK-1 type antibodies (Kruse et al., 1984, 1985; O'Shannessy et al., 1985; Willison et al., 1986; Fahrig et al., 1990; Harper et al., 1990; Lipford and Wright, 1991; Fredman et al., 1993) are inhibitors of adhesion involving CAMs. According to Schneider-Schaulies et al. (1990), recombinant (unglycosylated) P0 is as efficient as native P0 for adhesion. By contrast, Filbin and Tenekoon (1991, 1993) demonstrated that the integrity of the N-glycan of glycoprotein P0 is necessary for *homophilic* interaction, probably *«by stabilizing the homophilic binding site»*. However, as discussed below, the homophilic nature of this binding is questioned.

Another homophilic mechanism, but Ca^{++}-dependent, has been proposed for cadherins (Rutishauser, 1989; Takeichi, 1990; Geiger and Ayalon, 1992; Pouliot, 1992). It is proposed to take place through interactions between protein domains (Fig. 1b). But as in the case of CAMs, this hypothetical domain remains to be discovered.

Carbohydrate-carbohydrate interactions

Several types of carbohydrate-carbohydrate interactions have been described essentially Ca^{++}-dependent. As a well documented mechanism is the species-specific aggregation-factor of sponges, a proteoglycan sharing relatively short and complex oligosaccharide chains (Mr 6000) containing fucose, mannose and glucuronic acid (Misevic and Burger, 1990a,b, 1993). Recently, it has been proposed (Needham and Schnaar, 1993a) that the glycolipid SSGL-1 (Fig. 2b), sharing the HNK-1 glycan epitope, is involved in a Ca^{++}-dependent homophilic interaction (Fig. 1d). But since the glycolipid is a ligand for L- and P-selectins (Needham and Schnaar, 1993b), the above interaction may be well representing carbohydrate-lectin interaction. A calcium-dependent homophilic interaction is proposed for the glycolipid Le^x (Fig. 2a; Eggens et al., 1989; Hakomori, 1992a) or GM_3 (Kojima and Hakomori, 1991).

Protein-protein heterophilic interactions

Calcium-dependent *heterophilic* mechanisms have been largely documented for *heterotypic* interactions (interactions between different cells or surfaces). This is the case for integrins and their respective ligands mainly involved in cell-matrix adhesion (Aota et al., 1991; Hynes, 1992; Albelda, 1993 Bosman, 1993). The molecules of the family of integrins are thought to recognize short peptide domains in their ligands (Fig. 1f). One of the most famous is RGD (Arg-Gly-Glu), but other candidates have been proposed like IKVAV (Federoff et al., 1993) or LHGPEILDVPST (Garcia-Pardo et al., 1990). Surprisingly, there is an increasing evidence that modulation of the carbohydrate composition of both integrins and of their ligands are influencing the adhesion process through an unknown mechanism (Chandrasekaran et al., 1991; Chammas et al., 1993; Federoff et al., 1993; Sriramarao et al., 1993). In addition, three component interactions, involving proteoglycans have been described (Iida et al., 1992).

Protein-carbohydrate heterophilic interactions

This mechanism has been widely documented from viruses to human (Monsigny et al., 1983; Barondes, 1984, 1988; Sharon, 1987, 1993; Geoffroy and Rosen, 1989; Leffler and Barondes, 1989; Leffler et al., 1989; Sharon and Lis, 1989; Cooper and Barondes, 1990; Lasky, 1991; Springer, 1991; Lasky and Rosen, 1992; Zanetta et al., 1992a,b; Bevilacqua and Nelson, 1993; Gabius et al., 1993; Mehrabian et al., 1993). The most commonly considered involves membrane-bound carbohydrate-binding proteins (lectins) on one cell playing a role in internalization of glycoproteins (Kawasaki and Ashwell, 1976; Chiacchia and Drickamer, 1984) or in adhesion with other cells (*heterotypic* recognition). Although lectins with different carbohydrate specificities have been involved (Schauer et al., 1990; Vandenberg et al., 1992), the overwhelming recent examples come from the discovery of family of selectins responsible for the specificity of homing of cells of the immune system and probably of some cancer cells (Monsigny et al., 1983; McEver, 1991; Springer, 1991; Durand, 1992; Lasky and Rosen, 1992; Bevilacqua and Nelson, 1993; Stoolman, 1993). These molecules, endowed with a Ca^{++}-dependent carbohydrate-recognition domain (C-type lectins; Drickamer, 1988, 1989, 1992), are binding with a high affinity complex oligosaccharides (Fig. 1h). The ligands are

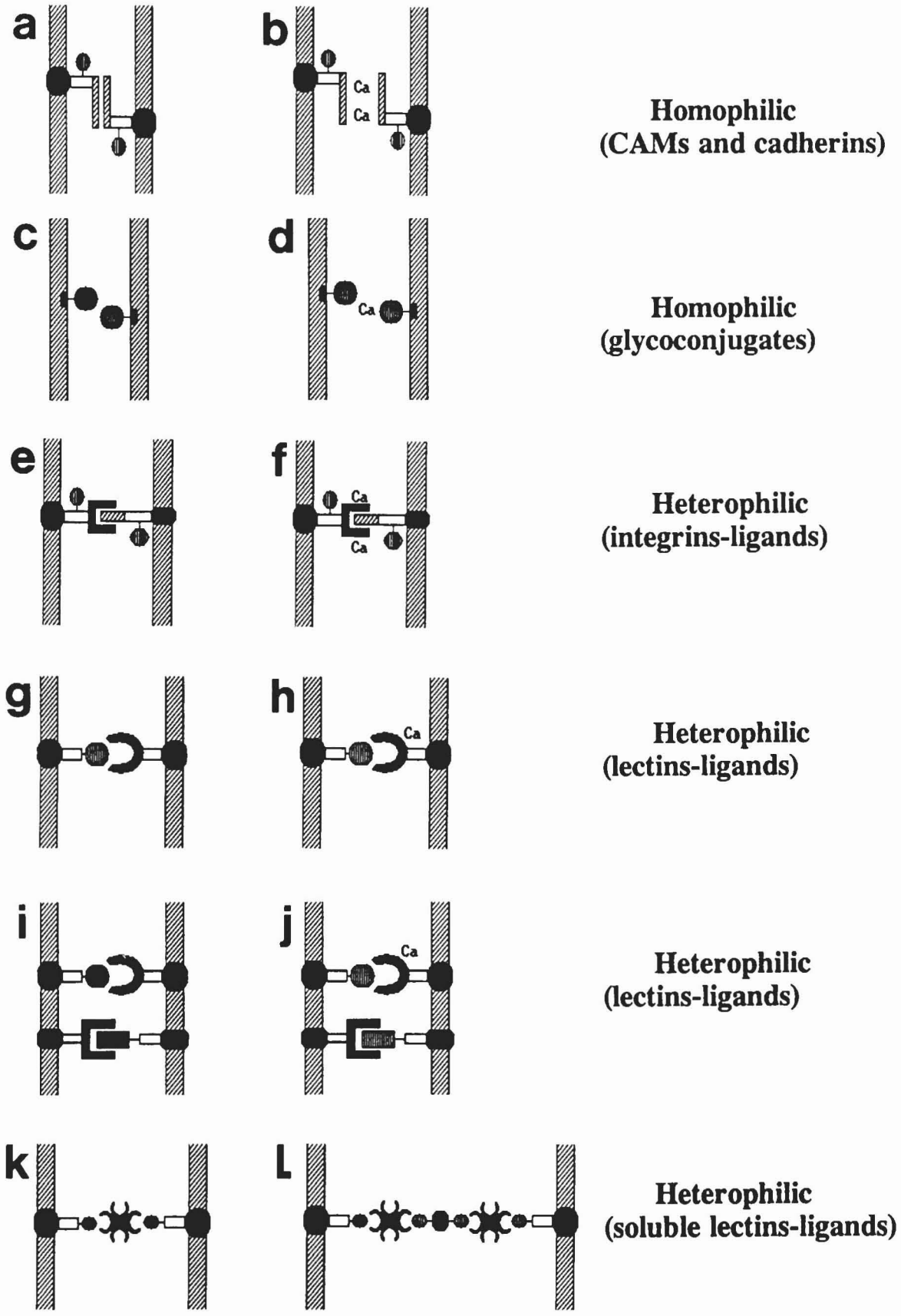


Fig. 1. Schematic representation of the adhesion mechanisms documented in the literature. a, c, e, g, i, k and l are calcium independent in contrast with b, d, f, h. **a and b.** Binding between peptide domains of identical molecules on two different cells. **c and d.** Binding between carbohydrate moieties of glycolipids. **e and f.** Protein recognition of small peptide domains on two different cells. **g. and h.** Protein-carbohydrate interaction between membrane-bound components on two different cells. **i and j.** Couples of protein-carbohydrate interactions on two different cells. **k.** Protein-carbohydrate interactions through soluble lectins and membrane-bound ligands. **l.** Protein-carbohydrate interactions through soluble lectin and both soluble and membrane-bound ligands.

sialyl-Le^x (Fig. 2a) and sialyl-Le^a on activated leukocytes or malignant cells for the endothelial selectin (E-selectin; Phillips et al., 1990; Handa et al., 1991; Polley et al., 1991; Larkin et al., 1992; Munro et al., 1992; DeFrees et al., 1993), sulfated and fucosylated O-linked glycans of the adressins or sialyl Le^x expressed on activated endothelial for the constitutively expressed leukocyte selectin (L-selectin; Spertini et al., 1991a,b; Green et al., 1992; Paavonen and Renkonen, 1992; Imai and Rosen, 1993; Imai et al., 1993; Sawada et al., 1993; VonAndrian et al., 1993). But other systems of cell recognition have been described like the interaction of the Ca^{++}-independent lectin R1 and its mannose-rich axonal ligands (Fig. 1g) during the synaptogenesis in the rat cerebellum (Dontenwill et al., 1985; Lehmann et al., 1993; Zanetta et al., 1985). There is still a few demonstration that the lectin and its ligands could be present *in vivo* on the same cell and, consequently, that this mechanism is also involved in *homotypic* cell recognition. The exceptions are the role of lectin R1 in myoblast fusion, probably interacting with Man8GlcNAc2 N-glycans of the myoblast surface at the time of fusion (Thomas et al., 1994), both *in vivo* and *in vitro*.

Such heterophilic interactions involving carbohydrates may be invoked for molecules suggested initially as involved in adhesion mechanisms through protein-protein interactions. For CAMs and cadherins, as for integrins and their ligands, it is well documented that heparin is a relatively good inhibitor of adhesive functions. Heparin-binding peptide domains are present (sometimes repeated) apparently interacting with specific oligosaccharide sequences (Dixit et al., 1984; Edgar et al., 1984; Cole and Glaser, 1986; Cole et al., 1986; Charonis et al., 1988; Pietu et al., 1989; Haugen et al., 1990; Rao and Kefalides, 1990; Guo et al., 1992; Hogasen et al., 1992; Novokhatny et al., 1992; Sobel et al., 1992; Underwood et al., 1992; Aukhil et al., 1993; Gee et al., 1993; Margalit et al., 1993; Mohri and Ohkubo, 1993; Pechik et al., 1993; Woods et al., 1993; Yurchenco et al., 1993). Since heparin is inhibiting a large number of adhesion mechanisms, it remains that lectin-like interactions are a possibility. However, it is not generally demonstrated that *in vivo* adhesion mechanisms of CAMs, cadherins and integrins and their ligands take place through a binding of the heparin-binding domains to heparin-containing molecules of the cell surface and/or the extracellular space. Such type of interactions has been suggested for the interaction of $\alpha 4\beta 1$ integrin with fibronectin involving also a chondroitin sulfate proteoglycan (Iida et al., 1992) and for N-CAM interacting with a cell surface heparan sulfate (Kallapur and Akeson, 1992; SanAntonio et al., 1993).

Soluble polyvalent protein-carbohydrate interactions

Other mechanisms involving oligosaccharides and carbohydrate-binding proteins emerged, based on the demonstration in many organisms, including vertebrates and mammals, of soluble and polyvalent lectins (Barondes, 1984, 1988; Sharon, 1987; Zanetta et al., 1987, 1992a,b; Jia and Wang, 1988; Leffler and Barondes, 1989; Leffler et al., 1989; Cooper and Barondes, 1990; Gabius et al., 1993; Sharon and Lis, 1989). At specific developmental stages of cells, these lectins may be externalized and, therefore, are supposed to make bridges between surface ligands, inducing both *homotypic* and *heterotypic* adhesion (Fig. 1k,l). But other mechanisms have been demonstrated. A well documented example is that of the L-14 (lactose-binding) lecting during myoblast fusion. This externalized dimeric lectin does not bind to ligands of the cell surface, but only to laminin externalized by myoblasts (Fig. 3). Consequently, L-14 inhibits the binding of the myoblasts to the laminin of the basal lamina (Cooper et al., 1991). For the Cell Syntactin Lectin CSL (Zanetta et al., 1987), a positive role in adhesion has been described. The polyvalent lectin (40 identical subunits) makes bridges between surface glycans of two cells establishing junctions (Fig. 1k) which can be dissociated by anti-CSL Fab fragments or carbohydrate competitors (Kuchler et al., 1988, 1989b,c, 1994a; Lehmann et al., 1990, 1991; Maschke et al., 1994). This mechanism can be involved either in homotypic (adhesion between identical cells, as demonstrated for cultured astrocytes (Kuchler et al., 1989c), oligodendrocytes (Kuchler et al., 1988), ependymal cells (Perraud et al., 1988; Kuchler et al., 1994a), C6 glioblastoma cells (Maschke et al., 1994), CHO cells (Lehmann et al., 1991)) or heterotypic (adhesion between different cells: axons and myelinating cells (Kuchler et al., 1989b), neurons and astrocytes (Lehmann et al., 1990)). But bridges can be made between the ligands at the surface of the same cell inducing a clustering and consequently a signal (Badache et al., 1994; Maschke et al., 1994; Zanetta et al., 1994b). Due to the high polyvalence of CSL, both mechanisms can take place at the same time, establishing a relationship between adhesion and signal transduction. However, in some cases, the situation appears as more complex since, in CHO cells, contacts take place through an intermediate layer (Fig. 1l; Lehmann et al., 1991).

Clustering of surface glycans and signal transduction

There is a large body of evidence that remodeling of the distribution of surface molecules is followed by generation of proliferative or differentiation signals. This has been largely documented using polyvalent compounds like antibodies to surface molecules or plant lectins, which have to be considered as exogenous clustering effectors for surface molecules (see for review Feizi and Childs, 1987). Similar data were found using chemical bi-functional agents (Cahill et al., 1993), inducing clustering of surface proteins. The polyvalence of these exogenous agents is indispensable since Fab

a)

Gal(β1--3)GlcNAc(β1--3)Gal(β1--4)Glc(β1-- **Le^a**
└(4--1α)Fuc

Gal(β1--4)GlcNAc(β1--3)Gal(β1--4)Glc(β1-- **Le^x**
└(3--1α)Fuc

NeuNAc(α2--3)Gal(β1--4)GlcNAc(β1--3)Gal(β1--4)Glc(β1-- **Sialyl-Le^x**
└(3--1α)Fuc

b)

SO4(--3)GlcUA(β1--3)Gal(β1--4)GlcNAc(β1--3)Gal(β1--4)Glc(β1--) **SSGL-1**

SO4(--3)GlcUA(β1--3)Gal(β1--4)GlcNAc(β1--3)Gal(β1--4)GlcNAc(β1--3)Gal(β1--4)Glc(β1--) **SGGL-2**

c)

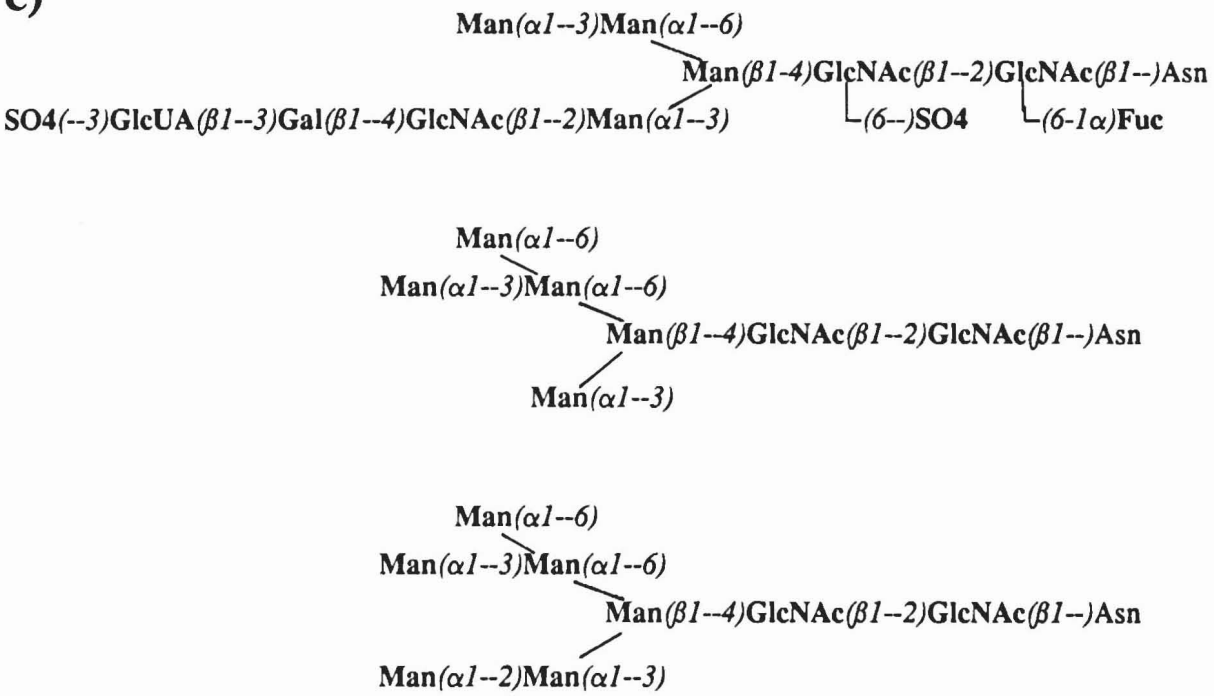


Fig. 2. Structures of glycans considered as important in cell adhesion processes. **a.** Structures of oligosaccharides representative of Lewis^a (Le^a) and Lewis^x (Le^x) carbohydrate antigens and the sialylated form of Le^x (sialyl-Le^x). **b.** Structures of oligosaccharides of glycolipids having the HNK-1 epitope. **c.** Structures of the N-glycan of P0 sharing the HNK-1 epitope, of Man5GlcNAc2Asn produced by N-acetyl-glucosaminyl-transferase I-deficient mutant CHO cells, and Man6GlcNAc2 ligand of CSL and recognized by L3 monoclonal antibodies.

fragments induces opposite effects. Clustering of surface molecules can be obtained also by adhesion of cells on surfaces coated with a sufficient density of adhesion molecules, as efficient as the clustering by polyvalent compounds (Kornberg et al., 1991, 1992; Lipfert et al., 1992). When submitted to such clustering, cells undergo a series of events characteristic of signal transduction (changes in calcium concentration or in c-AMP levels, specific phosphorylations on tyrosine or other residues, translocation in PKC, etc.) varying upon the cell type. These mechanisms have been extensively studied for a large variety of normal and malignant cells, showing both common and specific features. Nevertheless, the importance of these surface events was, in part, neglected for a certain time in favour of intracellular (cytoplasmic and nuclear) mechanisms. An increasing interest was directed to oncogenes (Bishop, 1983, 1985; Varmus, 1984; Ratner et al., 1985; Jove and Hanafusa, 1987; Kvanta et al., 1992), since transfection of cells with their genes were producing extremely important changes in the behavior of cells, not only increased proliferation but also, sometimes, cell differentiation (Haluska et al., 1987; Jove and Hanafusa, 1987; Holmes et al., 1992; Wen et al., 1992; Marchionni et al., 1993). Similarly, the increasing number of activators or inhibitors of intracellular enzymes or receptors led to the concept that surface events were only marginal in regulating cell differentiation or proliferation. However, recently, the relative importance of surface mechanisms has been seriously re-examined in different experimental systems. The discovery of the focal adhesion tyrosine kinase (p125(FAK); Kornberg et al., 1992; Zachary and Rozengurt, 1992) was an important finding, which re-actualized the old concept that contact between cells may regulate their behavior.

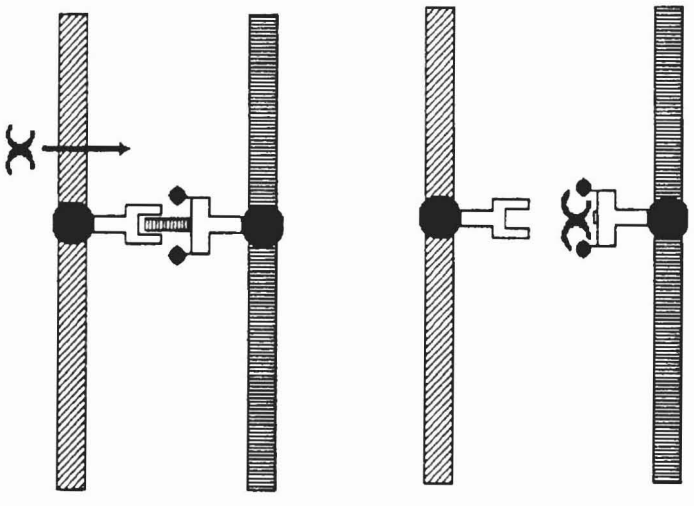


Fig. 3. Schematic representation of the effect of the externalisation of L-14 muscle lectin. When externalized, L-14 binds to laminin oligosaccharides (•) and inhibits the attachment of the cell to the laminin substratum (postulated as integrin-ligand interaction).

Clustering of surface molecules and signal transduction

Using polyvalent antibodies, it was shown that clustering of some surface molecules can generate signals. Apparently, they belong to different types of molecules including members of the superfamily of immunoglobulins (Nitta et al., 1989; Schuch et al., 1989), integrins and ligands (Chandrasekaran et al., 1991; Kornberg et al., 1991, 1992; Burridge et al., 1992; Lipfert et al., 1992; Huang et al., 1993; Schwartz, 1993), glycosyl-phosphatidylinositol anchored proteins (Kay et al., 1991; Represa et al., 1991; Seaman et al., 1991; Cinek and Horejsi, 1992; Suzuki et al., 1992, 1993; Draberova and Draber, 1993; Vivien et al., 1993), some of them involved in adhesion mechanisms, the other not. The signals were strongly reinforced when the first polyvalent antibody was further clustered by addition of a second anti-Ig antibody (Fig. 4a). Polyvalent plant lectins plays differential roles depending on their carbohydrate binding specificities. ConA, PHA, PWA are able to generate proliferative signal for leukocytes, whereas lectins specific for lactosides are generally inhibitory. For the latter, conclusions on their role should be carefully drawn since some of them contain a non-lectin subunit, which may have its one effect. With lectins generating proliferative signals, it is also difficult to conclude to one specific mechanism since they are binding, and consequently potentially clustering, a large number of molecules at the cell surface (Fig. 4b). Thus, also experiments performed with anti-carbohydrate antibodies and lectins are indicative that surface carbohydrates may be important for clustering of surface components, they do not provide evidence that glycans are actually involved in clustering of surface molecules *in vivo*. In contrast, experiments performed with N-glycosylation inhibitors are more convincing. Several papers (see for review Bowling et al., 1989, 1991) reported that treatment of lymphocytes with swainsonine potentialized the activation by ConA, whereas such effect was not obtained in cells treated with castanospermine. The latter produces immature $GlcMan_9GlcNAc_2$ N-glycans which are binding to ConA. Swainsonine is producing hybrid type N-glycans which are also binding to ConA. Thus it was expected that both N-glycosylation inhibitors would produce increased efficiency of activation by ConA. Thus, it appeared that clustering of surface molecules by ConA is not sufficient for activation, but that the nature of the N-glycan is fundamental. According to the adhesive mechanisms discussed before, it can not be assumed that the correct N-glycan is necessary for stabilizing homophilic binding sites of surface glycoproteins (Filbin and Tenekoon, 1991). In contrast, these experiments suggest the involvement in clustering of endogenous surface lectins (Fig. 4b).

Clustering of surface complexes

There is convincing data reporting that cell-surface

molecules involved in the generation of surface signal are not present at the surface as isolated molecules. The most documented examples are concerned with the T-cell receptor (Altman et al., 1990; Bierer and Burakoff, 1991; Beyers et al., 1992; Izquierdo and Cantrell, 1992; Isakov, 1993) and the B-cell receptor (Cushley and Harnett, 1993). Immunoprecipitation using antibodies to one constituent does not allow the isolation of the individual antigen, but of several molecules firmly bound the one to the other. Similarly, it has been shown that surface glycosyl-phosphatidylinositol anchored glycoproteins are firmly (but not covalently) associated with other proteins, particularly tyrosine kinases (Low and Saltiel, 1987; Cinek and Horejsi, 1992; Draberova and Draber, 1993). These complexes may involve microfilaments (Carraway et al., 1993; Haimovich et al., 1993; Pumiglia and Feinstein, 1993). But, surface molecules are part of complexes which need correct

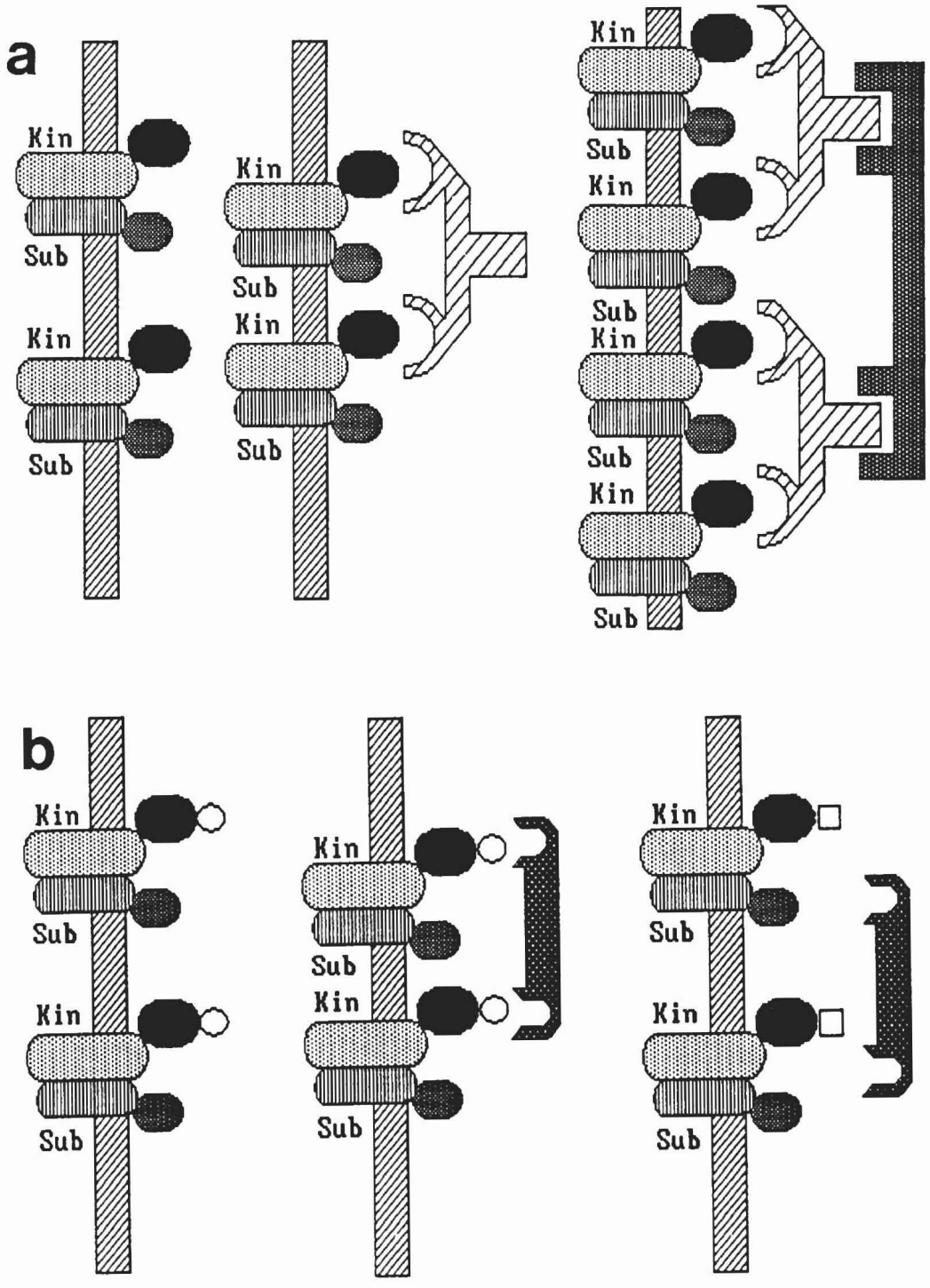


Fig. 4. Schematic representation of the effect of clustering on signal transduction (here phosphorylations). **a.** Effect of polyvalent antibodies: the cell surface complexes, initially separated are linked by a bivalent IgG. The kinase of one complex (kin) comes in vicinity of its substrate (Sub). The addition of a second antibody increases clustering of complexes and consequently signal transduction. **b.** The same type of effect is induced by polyvalent lectins interacting with specific glycans (•). When the structure of the glycan is modified, the lectin does not behave anymore as a clustering agent.

assembly for signal transduction (Altman et al., 1990; Beyers et al., 1992; Izquierdo and Cantrell, 1992). The experiments performed with polyvalent agents (antibodies and lectins or chemical agents) clearly showed that isolated complexes are frequently not sufficient for generating and transducing signals. Thus, the concept emerged that clustering of complexes is a fundamental step for generation of signal. However, such signal generation and transduction can also be obtained using monovalent ligands. The well known examples are the action of interleukins and insulin, where binding of the monovalent ligand to its surface receptor is inducing a signal, suggesting that two types of different mechanisms could take place.

Signal transduction generated by receptors for monovalent ligands and by intracellular molecules

The mechanism of generation of signal by interleukin 2 (IL-2) receptor and the insulin receptor have been extensively studied and provided interesting conclusions. The site directed mutagenesis of the four potential N-glycosylation sites of the ß-subunit of the insulin receptor (replacement of the four Asn by Gln) yields a receptor which normally expressed at the cell surface, with α- and ß-subunits normally associated and fixing insulin with the same kinetics as the non-mutated receptor (Leconte et al., 1992). However, the mutated receptor is not able to induce any of the normal response to insulin binding (tyrosine-phosphorylation of the α-subunit, uptake of glucose, etc.). Since it has been shown that tyrosine-phosphorylation is a cross-phosphorylation between different receptor molecules, the data were interpreted as a role of N-glycans of the ß-subunit in the clustering of insulin receptor molecules. Furthermore, using inhibitors of N-glycan processing (Duronio et al., 1988), it was shown that when cells are treated with inhibitors of glucosidases I or II, yielding immature N-glycans, the insulin receptor behave similarly (Fig. 5). In contrast, a totally functionally receptor was obtained treating cells with inhibitors of α-mannosidase I and II (Fig. 5), yielding oligomannosidic and hybrid type N-glycans, respectively. Thus, insulin receptor clustering appears as dependent of special N-glycans on its ß-subunit. Such experiments suggest that cell surface lectins specific for oligomannosidic and hybrid type N-glycans is necessary for this clustering. The same conclusions are suggested by experiments performed using interleukin 2 activation of lymphocytes (Bowlin et al., 1989), since swainsonine and not castanospermine is potentializing the activation. Since clustering of the IL-2 receptor is necessary for transducing signal upon binding of IL-2, the same pattern emerged: N-glycans, and more precisely oligomannosidic and hybrid type N-glycans are necessary for clustering of surface molecules. As dicussed before, the same N-glycans also appear as fundamental for adhesive properties.

The induction of proliferative and differentiation signals can be induced by modifications of molecules intracellularly localized. This is obtained every time when second messenger systems are modified (Ca^{++}, cAMP, cGMP, etc.). The discovery of phorbol esters as activator of protein kinase C initiated a large domain of research on the intracellular pathways, from the surface to the nucleus. However, modulation of intracellular signal transduction pathways also induces surface modifications. This has been particularly well documented for cells of the immune system. Activation of leukocytes by phorbol esters induces a rapid increase in the adhesive properties of the cells. Whatever the activation signal, one of the earliest events is the synthesis or externalization of molecules involved in adhesion. This is concerned with adhesion between circulating cells (Brod et al., 1990; Bjorck et al., 1993; Cirulli et al., 1993; Clark, 1993; Elenstrom-Magnusson et al., 1993; Engel et al., 1993; Galea et al., 1993; Parish et al., 1993; Rabin et al., 1993; Ruffini et al., 1993; Vythdreese et al., 1993; Wyss-Coray et al., 1993) or between circulating cells and endothelial cells (Buckle and Hogg, 1990; Carlos et al., 1990, 1991; Chin et al., 1990; Filhaber et al., 1990; Issekutz, 1990; Singer, 1990; Thornill et al., 1990a,b; Kuhlman et al., 1991; Damle et al., 1992; Vanhuijsduijnen et al., 1992; Weller et al., 1992; Brizzi et al., 1993; Derossi et al., 1993; Fries et al, 1993; Taub et al., 1993; Turunen et al., 1993; Valittuti et al., 1993). The rule deduced from these experiments is that a very short time stimulation (taking place in a period of ten seconds) induces long lasting effects. This suggests that the initial stimulation is inducing the synthesis of molecules which relays the initial signal. This occurs similarly to extracellular stimulation by monovalent and polyvalent agents. Since adhesive properties of cells are changed before induction of proliferative signal (Nash and Mastro, 1993), it is suggested that molecules involved in adhesion and clustering are the first which are newly synthesized and play a role of amplifier of the initial signal.

Soluble lectins in adhesion and cell proliferation mechanisms

Up to day a few number of lectins have been considered in cell adhesion and cell proliferation mechanisms: the lactose binding proteins of the families of L-14 and L-29 (or CBP 35) and the mannose-binding protein CSL. The class of heparin-binding proteins, which includes a large variety of growth factors, will not be considered here, since there is no clear evidence that they are actually playing their roles as lectins (Kamo et al., 1986; Kohnke-Godt and Gabius, 1989; Ceri et al., 1990; Eloumami et al., 1990; Gabius et al., 1991a; Bourin and Lindhal, 1992; Hulmes et al., 1993), although recent studies indicate that bFGF has a high affinity for a hexasaccharide (Tyrrell et al., 1993).

Lactose-binding lectins

Lactose-binding lectins have a very wide distribution

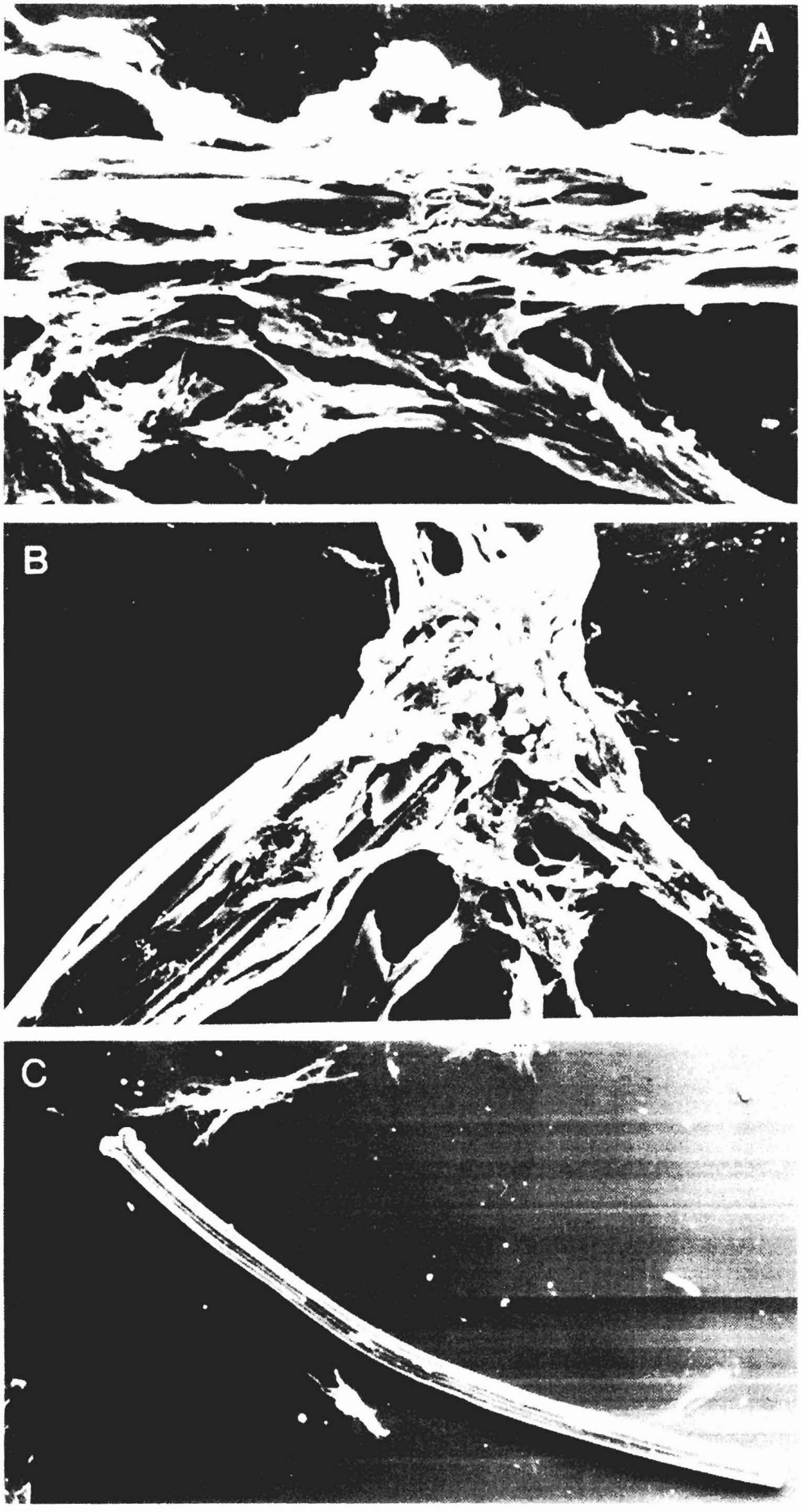


Fig. 5. Adhesion of Schwann cells to covalently coated cotton fibers with different molecules potentially involved in cell adhesion. **A.** CSL coated fibers; **B.** P0 coated fibers; **C.** Fibers coated with the MAG from adult rat myelin (not interacting with CSL). Note that Schwann cells are adhering to CSL and P0 fibers but not to MAG fibers. After one week in culture, cells are proliferating rapidly.

and phylogenetic studies show extensive conservation of these molecules during evolution. Up to 75% homologies are found betwen lactose-binding proteins of electric organ of the eel or of xenopus and mammalian lectins. These lectins are named S-type lectins since they have a characteristic carbohydrate recognition domain, which is conserved during evolution. Their activity is not Ca^{++}-dependent and is very sensitive to oxidative agents, probably because of an essential cysteine residue. They are present in most vertebrate tissues (muscle, lung, kidney, heart, brain; Leffler and Barondes, 1989), although they present cellular specificities and that isolectins are present in some cells, specifically (Leffler et al., 1989). Although they have been largely studied from a structural point of view, data on their involvement in cell adhesion processes are relatively rare. When isolated from various tissues these lectins behave as dimers formed of subunits around 14 kDa (therefore L-14) or 29 kDa (therefore L-29) (Barondes, 1984, 1988; Cerra et al., 1985; Caron et al., 1987, 1990; Sparrow et al., 1987; Bladier et al., 1989, 1991; Leffler and Barondes, 1989; Massa et al., 1993; Mehrabian et al., 1993; Oda et al., 1993) or carbohydrate-binding protein 35 (Crittenden et al., 1984; Moutsatsos et al., 1986, 1987; Laing and Wang, 1988; Wang et al., 1992; Agrwal et al., 1993; Knibbs et al., 1993). Consequently, the isolated lectins are able to induce agglutination of a large number of cells with various efficiencies (Joubert et al., 1987a,b). However, since the large majority of the lectin is intracellular, it not ascertained that when cell are synthesizing the lectin, the latter is actually involved in cell adhesion processes. Aggregation of cells by exogenously added lectins may be an *in vitro* artefact, until the demonstration of externalization of the lectin and its participation to the formation of bridges between cell surface ligands is provided *in vivo*. For example, lactose-binding lectins are not externalized, in the developing rat brain, the areas of synapses (as shown by electron microscopy; Joubert et al., 1989; Kuchler et al., 1989a) although light microscopy suggests an involvement in contacts between neurons. For the brain L-14 lectin, an interaction with actin has been demonstrated (Joubert et al., 1992). It is noteworthy that the dendritic spines of neurons where the lectin is accumulating is rich in actin-containing filaments, suggesting a co-transport of the two molecules. In contrast, the lectin is found at the surface of growing axons, suggesting, here, a role in cell adhesion (Kuchler et al., 1989a). This appears also for sensory neurons of the rat dorsal root ganglia (Regan et al., 1986; Dodd and Jessell, 1988; Hynes et al., 1989) since lactose-binding lectins and lactosides are present in subsets of neurons, accrediting the view that axon targeting may be regulated by interactions of the lectins with endogenous glycoconjugates.

The L-14 lactose-binding protein in myoblast fusion

An extracellular role of the L-14 has been demonstrated in C2C12 myoblast cell cultures at the period of fusion (Cooper et al., 1991). The L-14 is externalized at a period preceding myoblast fusion. As clearly demonstrated, the externalized lectin does not interact with potential ligands at the cell surface, but only with laminin of the extracellular matrix produced by the cells (Fig. 3). This specificity is surprising since potential ligands are present at the surface of myoblasts at this period, as demonstrated with galactoside-specific plant lectins. In fact, the binding to laminin appears to take place through Galα1-3 residues found on triantennary N-glycans of laminin, and laminin only (Marschal et al., in preparation). The explanation for this specific binding is probably the very strong differences in affinity for the N-glycans of laminin and the glycans (N-glycans or glycolipids, including gangliosides (Caron et al., 1993)) at the surface of myoblast. Although a positive effect in adhesion was expected, the action of the L-14 is actually to inhibit the binding of cells to laminin, rendering cells free to move and fuse. This specific interaction of lactose-binding proteins with laminin is not restricted to myoblasts but has been observed for other cells either directly or using galactose-containing neoglycoproteins (Kieda and Monsigny, 1986; Lotan and Raz, 1988; Gabius et al., 1991b; Ochieng et al., 1992).

Lactose-binding lectins and cell proliferation

In several systems lactose-binding lectins L-14, L-29 and CBP-35 are expressed at the cell surface and are often over-expressed in malignant cells (Roff and Wang, 1983; Kieda and Monsigny, 1986; Raz et al., 1986, 1990; Lotan and Raz, 1988; Lotan et al., 1989; Hass et al., 1990; Gabius et al., 1991b; Hamann et al., 1991; Irimura et al., 1991; Lotan, 1991a,b; Yamaoka et al., 1991). As bivalent compounds, it was expected that they will induce clustering of cell surface molecules and consequently will generate specific signals for cell proliferation or differentiation. However, the direct effect of these molecules and/or of the Fab fragments prepared from their specific antibodies on cell proliferation and on signal transduction was not studied into details. Suggestions were sometimes provided that endogenous lactose-binding proteins could be mitogenic. Activation of mast cells could be obtained using a galactose-specific lectin recognizing the IgE receptor (Frigeri et al., 1993). Transfection of poorly metastatic cells with the gene of the CBP-35 enhances their metastatic potential (Raz et al., 1990; Hamann et al., 1991; Lotan, 1991a,b; Yamaoka et al., 1991). However, since this molecule is also found in the nucleus, the question remains to know if the increased metastatic potential issues from nuclear or surface action of the lectin. Although these data are of importance, it is still not sure that the endogenous lactose-binding lectins externalized by the cells are capable of modulating normal and malignant cell proliferation. From a comparative study of the effects of differentiation

inducing agents on the expression of lactose-binding lectins in many malignant cell lines (Lotan and Raz, 1988; Lotan et al., 1989; Ohannessian et al., 1989; Lotan, 1991a,b), it appears that the cell type-specific lectins are differently affected, in a cell type-dependent scheme. No correlation was found between lectin expression and proliferation or differentiation. The absence of knowledge of the endogenous ligands of these lectins is impeding further interpretations. It is expected that the action of a ß-galactoside binding lectin would take place after binding to its ligands (surface, cytoplasmic, nuclear or extracellular matrix). Thus it is of primary importance to identify these molecules, as well as their localization. As it is known that the patterns of glycosylation of the various cells (normal or malignant) are generally specific of the cell type (Rademacher and Dwek, 1989; Ashford et al., 1993), different ligands coupled to different signal transduction mechanisms could provoke cell-specific response. In some cells, ligands of lactose-binding protein are present, which are over-expressed in malignant transformation. This is the case for the α-subunit of VLA-3 (Tsuji et al., 1990, 1991), a molecule of the integrin superfamily. Whether, glyco-biological interactions between this ligand (integrins are involved in signal transduction) is participating to cell proliferation or other mechanisms remains to be determined.

The Hodgkin's cell lectin

The Hodgkin's cell lectin has been characterized as a galactose-N-acetyl-galactosamine-specific lectin expressed at the surface of a major non-neoplastic type of lymphocytes found in Hodgkin's disease. Carbohydrate-binding and immunological properties suggested that this lectin is structurally related to the hepatic asialoglycoprotein receptor (Kawasaki and Ashwell, 1976). It was suggested that two forms of the lectin are occurring, one membrane-bound molecule and a soluble one, both playing a role in activation of normal lymphocytes (Paietta et al., 1989). The inhibition of the activation by desialylated glycoproteins or treatments of lymphocytes with ß-galactosidase suggested that galactose-specific lectins could induce lymphocyte activation. But direct evidence that the purified Hodgkin's cell lectin is involved is lacking. Similarly, inhibitions by polyclonal antibodies need caution before interpreting the data.

The mannose-binding lectins

Two families of mannose-binding lectins have been described, which do not present immunological analogies (unpublished data). They correspond to the family of the mannose-binding proteins (MPB; Kawasaki et al., 1978, 1983; Mori et al., 1983) and that of the cell syntactin lectin (CSL; Zanetta et al., 1987). The molecules of the family of MBPs are Ca^{++}-dependent mannose-binding proteins which have been extensively studied (Oka et al., 1987, 1988; Childs et al., 1989) and found in the serum and in hepatocytes of several species. The role of serum MBP in complement activation has been demonstrated (Ikeda et al., 1987; Ohta et al., 1990). As another physiological role of the molecules homologous to MBPs, are the fixation of some viruses and bacteriae (Kawasaki et al., 1989). However, the MBPs have not been considered as adhesion molecules in the liver, although a few number of glycoprotein ligands have been detected (Mori et al., 1988). No data on the potential role of MBPs in adhesion and cell proliferation and differentiation are available.

The mannose-binding lectin CSL constitutes a family of molecules with a relatively large distribution in vertebrate tissues, although it is absent from fibroblasts, endothelial and muscle cells. The different members of 31.5 kDa, 33 kDa, 43 kDa, 55 kDa and 75 kDa are presenting cellular specificity. Each compound is an homopolymer of about 40 identical subunits (Zanetta et al., 1987). Therefore, CSL behave as an agglutinin *in vitro* (Marschal et al., 1989) and as an adhesion molecule when used as a substratum (Fressinaud et al., 1988).

CSL as adhesion molecule

At certain stage of development of the nervous tissue, the CSL is externalized and makes bridges between cell surface ligands ensuring formation of contact between cells and, with maturation of the tissue, forming junctions. This has been demonstrated *in vitro* for oligodendrocytes (Kuchler et al., 1988), astrocytes (Kuchler et al., 1989c), ependymal cells (Perraud et al., 1988; Kuchler et al., 1994a), CHO and C6 malignant cells (Lehmann et al., 1991; Maschke et al., 1994). Experiments performed *in vivo* and *in vitro* suggest that CSL is involved in neuron migration along astrocytic processes (Lehmann et al., 1990), stabilization of myelin structure (Kuchler et al., 1989b) and contact between axons and myelinating cells. The *in vivo* experiments consisting in injecting in the brain ventricles of adult rats neoglycoproteins of various carbohydrate specificities (including mannose; Kuchler et al., 1994a) and different ascite fluids (including anti-CSL; Kuchler et al., 1994b) indicate that the ependymal tightening (ependymal cells are lining brain ventricles) depends only on interactions of CSL with its surface ligands. In the same experiments, it was shown that anti-CSL antibodies were penetrating into the brain and provoke demyelination in deep white matter, analogous to that observed in multiple sclerosis patients. This finding open new perspective in the field of multiple sclerosis since more than 96% of the multiple sclerosis patients showed anti-CSL antibodies in their cerebro-spinal fluid (1388 samples analyzed; Zanetta et al., 1990a, 1994a).

Endogenous ligands of CSL

The endogenous high-affinity ligands of CSL consist in most normal cells in a discrete population of

glycoproteins, generally present in low amount. In rat immature neurons, the P31 glycoprotein (Zanetta et al., 1978; Nédelec et al., 1992) and probably Thy-1 are the major CSL ligands. In human T and B cells, the major ligands of CSL are CD3 and CD24, respectively, and unidentified low Mr glycoproteins (Zanetta et al., 1994b). In proliferating oligodendrocytes and Schwann cells, the major ligand of CSL corresponds to the early form of the myelin-associated glycoprotein (Kuchler et al., 1989b; Badache et al., 1992). In myelin produced by these cells in adult, the ligands are low Mr glycoproteins in the central nervous system (Kuchler et al., 1990, 1991; Badache et al., 1992), P0 glycoprotein in the peripheral nervous system (Kuchler et al., 1989b; Badache et al., 1992). A very small portion of MAG, corresponding probably to the early MAG (Lai et al., 1987) is present in myelin preparation of adult rats, potentially involved in the maintenance of the contacts between axons and myelinating cells. For the MAG, the inhibition of CSL binding by HNK-1 type antibodies suggest that some N-glycans possessing glucuronic-3-sulfate group are the sites of CSL fixation. In P0, possessing one N-glycosylation site per molecule (Sakamoto et al., 1987), the ligand has probably the structure determined by Uyemura and Kitamura (1991) for the HNK-1 binding N-glycan of the P0 molecule (Fig. 2c), which as most P0 N-glycans is sulphated (Uyemura and Kitamura, 1991; Field et al., 1992). In contrast, most malignant cells have very high amount (10-100 fold more than normal cells) and number (around 100) of CSL ligands. In these cells, the profiles of the CSL ligands were virtually identical, independent on the tissue and species (Maschke et al., 1993). Indirect arguments suggest that the N-glycans binding to CSL in cancer cells are different from those found in normal cells.

Interaction of CSL with the glycoproteins of the superfamily of immunoglobulins

The interaction of CSL with members of the superfamily of immunoglobulins asks important questions on the molecular mechanisms involving these molecules in adhesion. Most transfection experiments designed for demonstrating homophilic protein-protein interactions were performed in CHO or C6 cells, containing CSL and expressing high amounts of N-glycans recognized by CSL (Lehmann et al., 1991; Maschke et al., 1993, 1994). In the two untransfected cell types, CSL is a major component of the adhesion system by interacting with its ligands (Lehmann et al., 1991; Maschke et al., 1994). Thus, all data previously published as evidence for homophilic interactions ca be re-interpreted in another way: transfection of these cells with the genes of CAMs increase the number of CSL ligands and, consequently, the CSL-dependent adhesion. This hypothesis provides an alternative explanation to data showing the fundamental role of specific N-glycans *«in stabilizing the homophilic binding site»* (Filbin and Tenekoon, 1991). Indeed, it was shown (Filbin and Tenekoon, 1991) that the increased adhesivity of CHO cells transfected with the gene of P0 is abolished in CHO cell mutants defective in N-acetyl-glucosaminyl-transferase I, consequently synthesizing $Man_5GlcNAc_2$ N-glycans (Fig. 2c). These N-glycans are not binding to CSL. Thus the interpretation of the results could be that since P0 synthesized by these mutant CHO cells are not binding to CSL, no adhesion occurs. In fact in these mutants, not only transfected cells are poorly adhesive but also the untransfected mutant which does not synthesize the CSL binding N-glycans. Recently, it was shown (Filbin and Tenekoon, 1993) that the involvement of P0 in homotypic adhesion is symmetrical, in other words, molecules of P0 on two cells in contact should have the right N-glycan for adhesion. It excludes the hypothesis (Schneider-Schaulies et al., 1990) that P0 also have a lectin-like activity. Although these experiments cannot provide a definite conclusion whether adhesion is homophilic (P0-P0 interactions) or CSL-dependent (bridges of the N-glycans of P0 by CSL), the data by Yazaki et al. (1992) obtained on C6 cells transfected with the gene of P0 cannot be interpreted in terms of homophilic interaction. Indeed, isolated glycopeptides from P0 (comprising a very short polypeptide chain attached to them) showed a very strong inhibitory effect on the increased adhesion of C6 cells transfected with the gene of P0. In contrast, these data fit perfectly with the demonstration that CSL and CSL-binding N-glycans are the key molecules in C6 adhesion mechanisms (Maschke et al., 1994). C6 cells cultured in the presence of inhibitors of N-glycosylation producing N-glycans not binding to CSL are not reaggregating and reaggregation of C6 cells producing CSL-binding N-glycans is completely inhibited by anti-CSL Fab fragments.

A particular attention was directed on the adhesive properties of the myelinating cells of the peripheral nervous tissue, the Schwann cells. During their differentiation, Schwann cells expressed at their surface CSL and, in high amounts, two members of the superfamily of immunoglobulins. In early stages (proliferation and beginning of myelination), MAG is the major surface glycoprotein. With differentiation (myelination), Schwann cells express very high amounts of P0, which is not expressed in early stages. Both molecules have some (not all) polypeptide chains binding to CSL because of developmental regulation of their N-glycans. During the early stages, most chains of MAG have CSL-binding glycans. Although MAG is increasing thereafter, CSL-binding MAG is decreasing. Furthermore, the CSL-binding MAG can be separated from the other forms according to their solubility properties (Badache et al., 1992). Of particular importance is that the MAG classically isolated from myelin is not CSL-binding, and shows virtually no adhesive properties (Schneider-Schaulies et al., 1990).

An experimental paradigm was elaborated to reduce the complexity of cell-cell adhesion by studying the binding of isolated Schwann cells to covalently

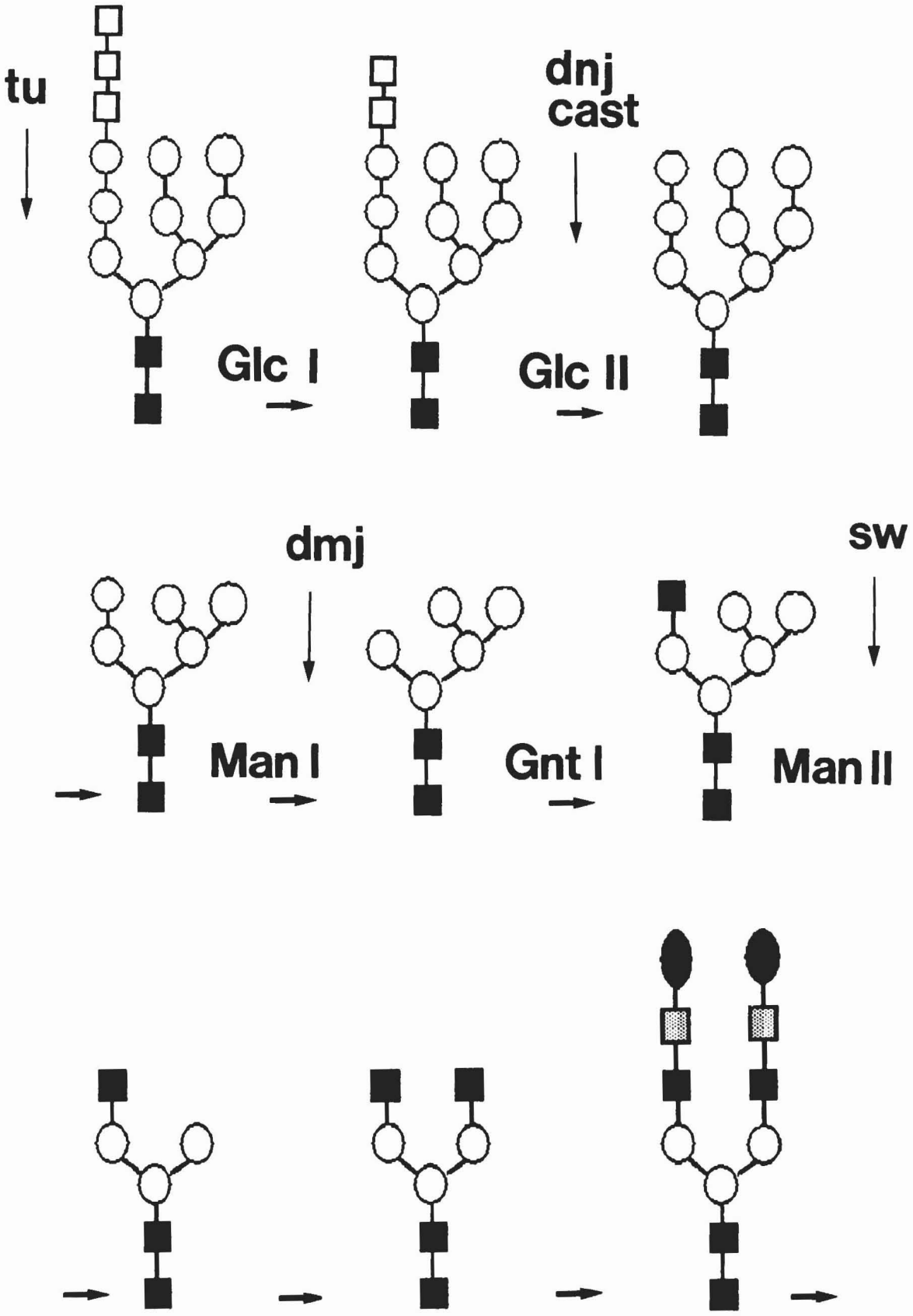


Fig. 6. Processing of the glycoprotein N-glycans. The Asn-linked Glc3Man9GlcNAc2 glycan is deglucosylated in the RER by glucosidases I and II (Glc I and Glc II), then by mannosidase I (Man I) in the Golgi apparatus. The addition of one GlcNAc residue by N-acetyl-glucosaminyl-transferase I (Gnt I) allows the action of the Golgi mannosidase II (Man II). Addition of other monosaccharides allows the formation of complex N-glycans. The glucosidase inhibitors deoxynojirimycin (dnj) and castanospermine (cast) produce N-glycans with terminal glucose (empty squares) residues. Deoxymannojiri-mycin, the inhibitor of mannosidase I, produces oligomannosidic N-glycans (mannose = empty circles). Swainsonine, the inhibitor of Golgi mannosidase II is producing hybrid type N-glycans with mannose in terminal position on one branch and the classical sequence N-acetyl-glucosamine (black squares)-Galactose (grey rectangles)-sialic acid)black ovals).

immobilized molecules. In order to avoid problems encountered when coating substratum (cells always reach the bottom of the wells by gravity and consequently adhere through unspecific mechanisms), we immobilized the molecules on cotton fibers, through which Schwann cells from new-born rat are passed at a very slow flow rate then washed. Cotton fibers are subsequently cultured in normal conditions. As shown in Fig. 6, Schwann cells are adhering to fibers coated with CSL and with P0 (and later are proliferating). In contrast, cells were not binding to the classical MAG. This simple experiment with short time contact between Schwann cells are immobilized molecules provide direct and indirect arguments favouring the role of CSL in adhesion. On the one hand, it shows that the early MAG (CSL-binding) of the Schwann cell surface is not able to interact with the classical MAG of cotton fibers. Although, the two MAG have the same extracellular domains, where the presumed homophilic binding sites are localized, they have no affinity the one for the other. On the other hand, it is still possible to hypothesize that binding is due to interactions between putative homophilic domains of MAG of Schwann cells and that of P0 on cotton fibers (which are supposed to be the same). Thus, since the two molecules have the right N-glycans, the homophilic binding sites should be preserved as suggested by Filbin and Tenekoon (1991). But, boiling of P0 fibers did not modify their binding capacity, suggesting that a correct protein conformation is not necessary. Finally, Schwann cells are binding to native CSL-coated cotton fibers and not to boiled CSL fibers. This demonstrates the role of CSL as an adhesion molecule and provides again a different interpretation which agrees with all the previously published data. The presence of CSL can explain the essential role in adhesion of N-glycans having the HNK-1 epitopes (Baba et al., 1986; Riopelle et al., 1986; Fahrig et al., 1990) and $Man_6GlcNAc_2$ epitopes (Kucherer et al., 1987; Fahrig et al., 1990) found on glycoprotein members of the CAM family. These N-glycans are the only one which appear to be involved in adhesion processes (Bollensen and Schachner, 1987; Kucherer et al., 1987; Fahrig et al., 1990; Griffith et al., 1992; Hall et al., 1993). Indeed, it has been demonstrated that CSL possesses a duality of specificity for some HNK-1 possessing N-glycans and for $Man_6GlcNAc_2$ N-glycans. This coincidence of specificity of adhesion, together with data reported above, suggest that N-glycan of CAMs, and possibly those of some other glycoproteins involved in cell adhesion (Schachner, 1989), are participating to adhesion as ligands of CSL.

CSL as a mitogen

In several systems, CSL behaves as a mitogen. This has been documented for oligodendrocytes (Fressinaud et al., 1988), Schwann cells (Badache et al., 1994), C6 glioblastoma cells (Maschke et al., 1994) and in lymphocytes (Zanetta et al., 1994b). In contrast, astrocytes, although CSL is important for adhesion mechanism, are not sensitive to CSL as a mitogen (Kuchler et al., 1989c). A comparative study was performed between Schwann cells and C6 glioblastoma cells, using inhibitors of N-glycosylation (tunicamycin, deoxynojirimycin, castanospermine, deoxymannojirimycin, swainsonine; Kornfeld and Kornfeld, 1985; Elbein, 1987), sulfation (chlorate; Poduslo, 1990), and traffic (monensin; Pimpaneau et al., 1989). The two types of cells were normally proliferating when CSL ligands are normally synthesized (deoxymannojirimycin and swainsonine), whereas proliferation was extremely reduced using inhibitors inducing absence of N-glycans (tunicamycin) or producing N-glycans not recognized by CSL (deoxynojirimycin and castanospermine). Furthermore, the proliferative activity of C6 cells cultured in the presence of the various inhibitors is related to the capacity of cells to reaggregate. Finally, anti-CSL Fab fragments are very potent inhibitors of both proliferation and adhesive properties of the C6 cells, as they are extremely potent inhibitors of the proliferation of Schwann cells (both basal proliferation and proliferation induced by axonal membranes).

A special attention was drawn by many researchers (Sobue et al., 1984; Sobue and Pleasure, 1985; Ratner et al., 1988; DeCoster and DeVries, 1989) to the neuronal membrane mitogen of Schwann cells, first discovered by Wood and Bunge (1975). The convergent conclusions were that the mitogen was adsorbed on ligands of the axonal membrane. Since it was solubilized by heparin and since inhibitors of synthesis of the addition of the glycosaminoglycans on the polypeptide chains of proteoglycans (ß-D-xylosides) inhibited the mitogenic effect, it was considered that the mitogen was bound to a heparan sulfate proteoglycan of the axonal membrane. Our recent data demonstrate that the axonal mitogen is CSL, bound to glycoprotein ligands of the axonal membranes, the major one being the P31 glycoprotein (Nédelec et al., 1992). Anti-CSL Fab fragments are inhibiting completely the mitogenic activity of axonal membranes and of the mitogen solubilized by heparin. The solubilizing action of heparin finds explanation since CSL is a heparin-binding protein. Similarly, it was shown (Ratner et al., 1988) that the membranes of cultured neurons treated with ß-D-xylosides preserved totally their mitogenic activity, whereas neuronal cultures treated with these inhibitors were loosing their mitogenic activity. The reason was that ß-D-xylosides provoked a stimulation of the synthesis and secretion of free glycosaminoglycans in the medium, which as extremely highly charged compounds interfered with binding of the mitogen to Schwann cells.

Differences in the behavior between C6 and Schwann cells were only observed for inhibitors of traffic (monensin, brefeldin A) and of sulfation (sulfate inhibits proliferation of C6 cells and the formation of CSL ligands whereas it does not inhibit the proliferation of Schwann cells and the synthesis of CSL ligands). These studies indicate that the intracellular traffic of the

glycoprotein ligands of CSL are different in C6 and Schwann cells and that the N-glycans recognized by CSL in the two cell types are different. But, these studies show that CSL and its ligands are essential molecules for C6 and Schwann cell proliferation. These observations are of interest since in most normal and malignant cells, it has been shown that N-glycosylation inhibitors showed the same pattern of effects: tunicamycin, deoxynojirimycin and castanospermine diminish cell proliferation. In contrast swainsonine and deoxymannojirimycin were ineffective in inhibiting proliferation. Since CSL and its ligands are present in most of these cells, it is suggested that the same mechanism as in C6 and Schwann cells is occurring. Therefore, the effects of N-glycosylation inhibitors are essentially due to the modulation of the structure of the CSL ligands at the cell surface.

CSL and signal transduction

In three cell types, a special focus was directed to mechanism of signal transduction. This was concerned with one mouse cancer cell line, the C6 glioblastoma cells, and with two types of normal cells, the rat Schwann cells and the human lymphocytes.

Oncogene products have been involved in proliferation of malignant cells. Therefore, it was of interest to see if N-glycosylation inhibitors, which reduced C6 cell proliferation, were modulating the expression of oncogene products. Throughout nine oncogene products analyzed, C6 cells expressed only *erbB2*, *abl/bcr* and *ras*. The two first oncogene products (substrate of tyrosine kinase and tyrosine kinase, respectively) were not modified using glycosylation inhibitors, suggesting that they were not involved in regulating C6 cell proliferation. A partial correlation was observed between the proliferative capacity of C6 cells and the expression of *ras* oncogene product. However, the relationship was not complete since *ras* was over-expressed in tunicamycin treated C6 cells, which have completely stopped their proliferation. The study of the activity and translocation of protein-kinase C (PKC, suggested to be essential for induction of proliferative signals) showed that all the inhibitors induced a translocation of PKC independent on the proliferative activity of C6 cells. Furthermore, the translocation of PKC induced by phorbol esters was not accompanied with increased C6 cell proliferation. These data suggest that C6 cell proliferation is totally independent upon PKC. Furthermore, the different inhibitors of N-glycosylation, sulfation and traffic were not modifying major tyrosine-phosphorylations detected using an anti-phosphotyrosine antibody on blots. Thus proliferation of C6 cells was apparently independent upon tyrosine-phosphorylations. In contrast, tunicamycin, deoxynojirimycin, castanospermine, monensin, brefeldin A and chlorate, which inhibit the synthesis or the expression of CSL ligands at the cell surface, are strongly inhibiting phosphorylations on other residues than tyrosine. It should be stressed that these phosphorylated proteins have an electrophoretic profile which is virtually identical to that of the CSL ligands. Since these ligands are common to most malignant cells, it may be hypothesized that loss of contact inhibition in most malignant cells is due to the over-expression of a large family of glycoproteins endowed with CSL-binding N-glycans and capable of generating proliferative signals through a specific type of phosphorylation.

CSL is a potent mitogen of Schwann cells. This was

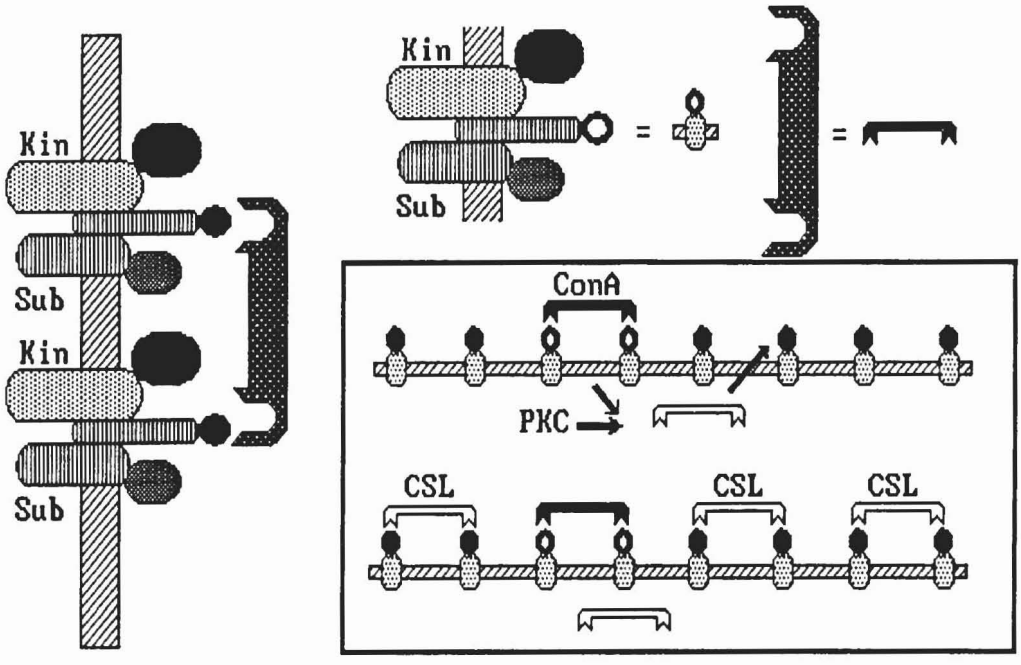


Fig. 7. Schematic representation of the activation process of lymphocytes by ConA or activator of PKC. The initial clustering of a few molecules involved in signal transduction by ConA induces the progressive synthesis and externalization of CSL which induces specific clustering of molecules involved in signal transduction. With activators of PKC, the massive synthesis and externalization of CSL allows a rapid intense signal transduction.

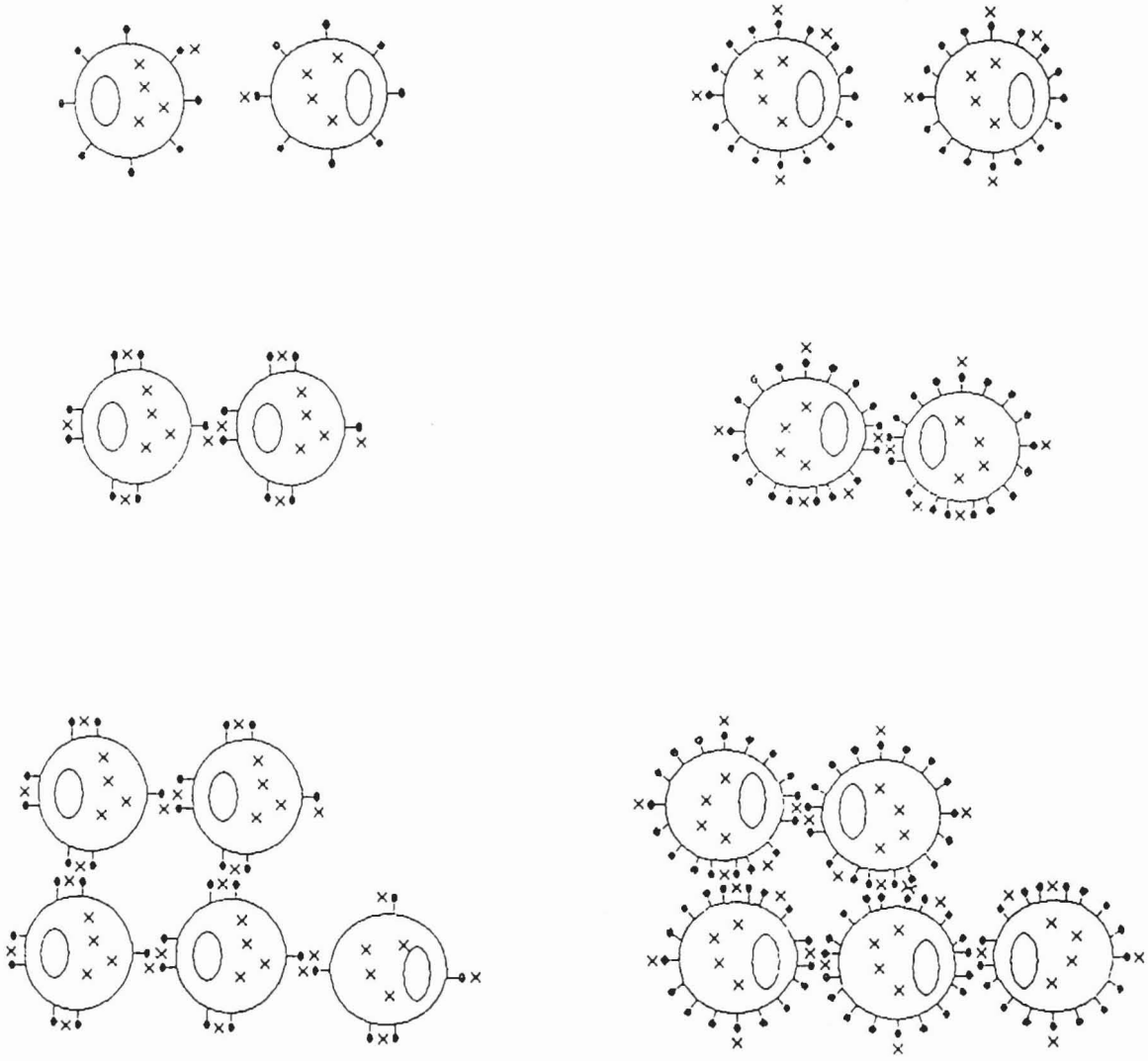
demonstrated by addition of exogenous CSL or by inhibition of Schwann cell proliferation by anti-CSL Fab fragments. The axonal mitogen for Schwann cells is CSL. Interestingly, CSL bound to axonal membranes appears as much more efficient as a mitogen than soluble CSL, suggesting that clustering of CSL on a cell surface is important for signal transduction. For all parameters so far studied, the situation is very similar to that observed in C6 cells. The pattern of inhibition of proliferation using inhibitors of N-glycosylation was the same. Differences were observed for the effect of chlorate, since ligands of CSL are not reduced, as well as the proliferative capacity of Schwann cells. In contrast with C6 cells, the different inhibitors are capable of modulating tyrosine-phosphorylations, establishing a correlation between Schwann cell proliferation, tyrosine-phosphorylation and expression of CSL ligands. Similarly, the effects of the various inhibitors on total phosphorylations follow the variations of the quantity of the CSL ligands and proliferative activity, as observed for the C6 cells. But, when considering the profiles of the phosphorylated proteins it appears as more complex than that of the CSL ligands. The early MAG, the major ligand of CSL, appears to be tyrosine-phosphorylated and phosphorylated on other residues, as other molecules which are not ligands of CSL. This suggests that in Schwann cells, cell surface proteins belong to complexes including MAG.

A special study was performed on human lymphocytes. The reason why this was investigated issued from previously published data (Bowlin et al., 1989, 1991) on the effect of inhibitors of N-glycosylations on activation induced by IL-2 or ConA. In the case of ConA activation, it was shown that ConA activation was potentialized in the presence of swainsonine but not in the presence of castanospermine (the two inhibitors are producing ConA-binding glycoproteins). The idea emerged that an endogenous lymphocyte lectin specific for hybrid type N-glycans produced in the presence of swainsonine was responsible for these differential effects. This corresponded to properties of the molecules of the CSL family. CSL is present at low level in quiescent lymphocytes as a single subunit of 31.5 kDa. Upon activation its maximal level is increased ten times within 2 h using activation by phorbol esters. The CSL is externalized and binds to a few ligands at the cell surface CD3 for T cells and CD24 for B cells (CD24 is extremely homologous to the P31 glycoprotein; Kay et al., 1991; Nédelec et al., 1992) and an unidentified low Mr ligand (18-20 KDa) apparently common to T and B cells. CSL appears as an extremely early expressed antigen. The expression of CSL at the cell surface is accompanied by signal transduction since CSL-dependent changes in tyrosine-phosphorylations and other phosphorylations are observed. When activation with phorbol esters is performed in the presence of anti-CSL Fab fragments, the pattern of tyrosine-phosphorylation shifted from the activated pattern to that of quiescent. This indicated that CSL was directly involved in this change in tyrosine-phosphorylation. The same pattern was observed for total phosphorylations. A direct evidence of the role of CSL in modulating phosphorylations associated with activation was obtained by incubating human lymphocytes with exogenously added CSL. In these conditions, total tyrosine-phosphorylation changes described above occurred within a period of 20 min. Therefore, both direct and indirect evidence is provided that CSL is an «amplifier» of signals necessary for activation.

There is a large body of evidence that activated lymphocytes have increased adhesion properties (Wagner et al., 1993). It was documented that activated T or B cells are able to activate quiescent T or B cells only by contact (use of membrane preparations). As recently described, N-glycans (Powell et al., 1993) and overall oligomannosidic N-glycans (Savage et al., 1993) are the keys in the mechanism of cross-activation. The same activation was obtained with membranes of cancer cells or astrocytes (Petrasch et al., 1992; Haegel et al., 1993; Peoples et al., 1993). Thus, considering that CSL is specific for hybrid and oligomannosidic N-glycans and that most cancer cells (or astrocytes) have high amounts of CSL ligands and CSL at their surface, it is strongly suggested that activations by contacts are mediated through CSL and its ligands. This offers a completely new understanding of cell contact and lymphocyte activation.

Modalities of action of CSL

As discussed before, there is increasing evidence that surface molecules involved in signal transduction are part of complexes. Exogenous polyvalent lectins and antibodies, as well as poly-functional agents inducing clustering of these complexes, are generally able to generate signals. A theoretical scheme involving surface clustering by polyvalent endogenous lectin has been proposed several years ago, which appears as a pioneer hypothesis now experimentally documented (Feizi and Childs, 1987). Our results propose that CSL is one of these key molecules in the clustering of cell surface glycoproteins and consequently is inducing signal intracellularly. The kinetic experiments performed on human lymphocytes indicate that tyrosine phosphorylation necessary for activation cannot be induced until CSL is clustering its surface ligands and that CSL synthesis appears as one of the earliest event during activation. The massive induction of CSL synthesis observed during lymphocyte activation has been also observed in aggregating brain cell cultures under the effect of EGF (Ténot et al., 1989; where CSL represents up to 5% of the total proteins of the aggregates, inducing a super-aggregation of aggregates). Under the specific agents, part of the CSL is externalized at the surface and binds and clusters its ligands. But, it also induces increased adhesive properties and probably more efficient clustering (adhesive clustering). This is suggested by the extreme efficiency of axon or myelin



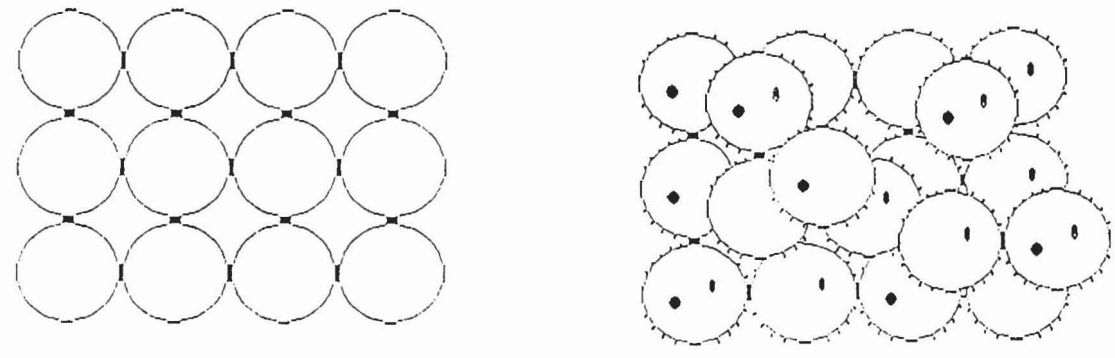


Fig. 8. Schematic drawing of the role of CSL (x) and of its ligands (•) in the loss of contact inhibition of malignant cells (right part) as compared to normal cells (left part). The plethora of CSL ligands allows the formation of multiple attachment sites, consequently, a three dimensional assembly of cells is possible in contrast with normal cells.

bound CSL as contrasted to the soluble CSL for mitogenic signals in Schwann cells (Badache et al., 1994) and of adhesion on CSL-coated substratum for Schwann cells and oligodendrocytes (here and Fressinaud et al., 1988). Depending on the cell types, different phosphorylation mechanisms occur, which trigger the proliferation signal. The pattern of phosphorylation appears as totally different in cancer cells (only one example) as compared with normal cells. In C6 cancer cells, tyrosine-phosphorylations are apparently not involved. Other phosphorylations are taking place and likely one on CSL ligands. Since these ligands are expressed in most malignant cells, it is reasonable to hypothesize that the same mechanism is occurring in the malignant cells over-expressing CSL ligands. In normal cells, tyrosine phosphorylations and other phosphorylations on a restricted number of proteins are taking place, the phosphorylated proteins being apparently (two examples) cell-type specific. The mechanisms by which these CSL-dependent phosphorylations are capable of inducing proliferative signals are unknown, but it can be postulated that it is involving classical pathways inducing cell proliferation.

CSL and its ligands in the loss of contact inhibition of malignant cells

Due to the general changes in the surface oligosaccharides during malignant transformation (see for review Hakomori 1985, 1989, 1992b), it was assumed that they were responsible for the loss of contact inhibition of cancer cells. The mechanism of regulation of cell proliferation and adhesion by CSL and its ligands proposes a comprehensive model for the loss of contact inhibition of the proliferation of malignant cells (Fig. 8). The major point is the over-expression of a special class of molecules in malignant cells, which allows the building of three dimensional structures, because the ligands (involved in the generation of proliferative signals) are abundant enough to be never saturated by the low amounts of CSL externalized by the cells. In cancer cells, there is still the possibility of more clustering and adhesion, in contrast with normal cells where the small amount of CSL ligands is limitative for clustering and adhesion. Furthermore, in most normal cells, the CSL ligands are transiently expressed at certain developmental periods and destroyed thereafter, persisting only in junctional areas with CSL. Regulation of the synthesis of these molecules is possible in normal tissues, but not in malignant cells, where the regulation of the expression of CSL ligands is suppressed. This hypothesis has some limitations because, some cells are not producing CSL, or CSL ligands or CSL and its ligands are not involved in signal transduction. The last example is concerned with astrocytes, which produce CSL and have CSL ligands. But, it seems that interaction of CSL with its surface ligands (heterogeneous in contrast with most normal CSL responsive cells) do not seem to be coupled to signal transduction pathways.

Nature of the CSL ligands

The studies of the CSL ligands over-expressed in most malignant cells indicate a virtual identity of their profiles independent on the tissue and species. This suggested that a certain class of molecules, not present in normal cells was expressed. The data concerning the CSL ligands in Schwann cells and C6 cells cultured with N-glycosylation inhibitors deoxymannojirimycin and swainsonine suggested that CSL binding N-glycans were essentially different from most N-glycans of the cells, since they did not become CSL ligands after these treatments. Thus, the hypothesis emerged that CSL ligands possess a specific polypeptide sequence necessary for the synthesis of these special N-glycans (Maschke et al., 1993). The analogy of the profile of the CSL ligands and protein phosphorylated on residues other than tyrosine in C6 cells, suggested that all these molecules had specific intracellular sequence for specific phosphorylations. Consequently, the hypothesis was made that this common sequence was intracytoplasmic, and plays two essential roles. The first is that of substrate for phosphorylation by specific kinases, the second is the intracellular trafficking of these glycoproteins. At the present time, our hypothesis is that this sequence is orienting CSL ligands to specific Golgi subcompartments where special transferases are present. The concept of addressing sequences is emerging recently (Ktistakis et al., 1990; Johnson and Kornfeld, 1992; Kodukula et al., 1992; Lowe, 1992; Najjar et al., 1993; Ozaki et al., 1993; Prill et al., 1993). Different products of alternative splicing are differently glycosylated (Bach et al., 1990; Hill et al., 1992). As mentioned before, the «early MAG», an alternative splicing variant of MAG is differently glycosylated as the «late MAG» and is CSL binding. These two molecules differ only by their intracytoplasmic domains (Lai et al., 1987). The recent production of a polyclonal antibody to one CSL ligand isolated from human K652 malignant cells (Maschke et al., 1994) provides interesting informations on this hypothetical sequence in malignant cells. Indeed, this antibody recognizes most CSL ligands in many malignant cells of various origins and in various human tumors, independently whether the CSL binding glycan is present or not. Furthermore, this antibody does not recognize CSL ligands in normal cells. Thus, although this sequence is still unknown, it appears that CSL ligands have in common a cancer-specific sequence (Maschke et al., 1994). The abnormal expression of this sequence may be the cause of the specific increase in CSL ligands, and consequently, loss of contact inhibition.

Conclusions on the roles of CSL

N-glycans have been shown to be important for various cell surface molecular or cellular events. This is the case for cell surface receptors, in which suppression of N-glycans (by tunicamycin or site-directed muta-

genesis) or change in their structures (by deoxymannojirimycin and castanospermine) does not modify binding of the ligands but uniquely signal transduction. Many cells (normal or malignant) respond with antiproliferative behavior using tunicamycin, deoxynojirimycin and castanospermine. The role of CSL as a clustering agent allows an explanation of these results, overall because many studies have emphasized that deoxynojirimycin and castanospermine are not modifying the synthesis, the traffic, the expression at the cell surface and the binding function of surface glycoproteins. These inhibitors only modify the carbohydrate structure of N-glycans. Similarly, deoxymannojirimycin and swainsonine, although they are not modifying the synthesis, the traffic, the expression at the cell surface and the binding function of surface glycoproteins are producing N-glycans with defined structures. Considering the known structures of the N-glycans produced in the presence of the different N-glycosylation inhibitors, it can be concluded that complex N-glycans (poly-antennary with terminal NeuNAcGalGlcNAc sequences) are not involved because they are not synthesized in the presence of swainsonine and deoxymannojirimycin. This is the same for poly-N-acetyl-lactosaminic sequences terminated or not by special epitopes like Le^x or sialyl-Le^x. Only hybrid type N-glycans and oligomannosidic N-glycans are involved in signal transduction. That means that structures shown in Fig. 2 are essential. But, according to Uyemura and Kitamura (1991), these glycans can be fucosylated on the first GlcNAc residue and sulfated on the second GlcNAc residue of the core of N-glycans. Ligands of CSL are found in these classes of N-glycans. For circulating cells, the presence of CSL bound to its surface ligands introduces a new understanding of adhesion mechanisms, since increased adhesivity of activated cells could, in part at least, be due to CSL. The same type of mechanism can be invoked in the formation of aggregates between activated leukocytes and malignant cells, the latter expressing high amounts of CSL ligands. A different role of CSL is suggested in multiple sclerosis. As previously described (Zanetta et al., 1990b), multiple sclerosis patients have very amounts of anti-CSL antibodies in the blood, suggesting a systemic autoimmune disease in which leukocyte CSL is the major target. Thus, immune complex formation is expected, responsible for increased aggregation of leukocytes in relapsing periods. The formation of demyelination plaques may be well originate from microthrombosis provoked by specific homing of leukocyte aggregates on specific endothelial cells found in individual developing the disease.

Conclusion and perspectives

The existence in mammalian cells of soluble polyvalent lectins with different carbohydrate-binding specificities opens new insights in the field of the regulation of adhesion and proliferation and/or differentiation of cells. For some of them at least, their intracellular over-expression and their externalization at specific period of the cell development has been documented. Their specific affinity for the carbohydrate moieties of cell surface glycoconjugates considered as adhesion molecules proposes a new understanding of adhesion mechanisms. These molecules are capable of clustering their cell surface ligands, inducing specific signal to the cells as proposed by the pioneer hypothesis of Feizi and Childs (1987). The function of surface glycans in the loss of contact inhibition of growth of malignant cells finds explanation, considering the roles of the CSL lectin as a clustering agent capable of inducing proliferative signals and as a bridging agent between cell surfaces. It can be imagined that the additional externalization of a lectin with a different carbohydrate specificity may interfere with the former mechanisms. The isolation of soluble lectins as active molecules becomes a primordial necessity for resolving the complexity of the regulation of contacts between cells in a tissue or between circulating cells and their target endothelium. It may be hypothesized that a soluble polyvalent lectin bound to its surface ligands is still capable of binding ligands on another cell and consequently participating to adhesion or homing. Tools are already accessible for these studies.

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