Differential potency of two crosslinking plant lectins to induce formation of haptenic-sugar-resistant aggregates of rat thymocytes by post-binding signaling

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Abstract To evaluate the significance of post-binding events for stable aggregate formation, the aggregation/dissociation of rat thymocytes initiated by two crosslinking plant lectins, namely concanavalin A (Con A) and *Solanum tuberosum* agglutinin (STA), were comparatively studied. Despite intimate cell contacts in the aggregates only Con A led to establishment of haptenic-sugar-resistant (HSR) complexes. The presence of inhibitor II of diacylglycerol kinase, a dual calmodulin antagonist/protein kinase C inhibitor (trifluoperazine), and a sulfhydryl group reagent (*N*-ethylmaleimide) impaired this process. The obtained results indicate that the formation of HSR cellular contacts is not an automatic response to lectin-dependent cell association. In contrast to STA, Con A binding elicits this reaction with involvement of diacylglycerol kinase, protein kinase C and/or calmodulin as well as thiol level perturbation, as inferred by the application of target-selective inhibitors.

Key words: Agglutinin; Aggregation; Cell adhesion; Lectin; Thymocyte; Trans-membrane signaling

1. Introduction

Elicitation of intracellular signaling processes by cytokines, growth factors and other stimulators affects not only the levels of an array of mediators but also the capacity of cells to be engaged in cell adhesion [1,2]. A recent illustration for the elicitor-dependent display of cell surface adhesion molecules are membrane lectins (e.g. P- and E-selectins) and integrins in the orchestrated cascade leading to firm cell adhesion between leukocytes and endothelium in inflamed area sections [3]. To delineate the precise nature of the processes involved which contribute to the final cell contact, we have turned to the investigation of model systems. Previously, we had found that the aggregation of different types of cells mediated by the galactoside-specific mistletoe lectin and mannoside-specific *Escherichia coli* agglutinin is a prelude to the formation of haptenic-sugar-resistant (HSR) intercellular contacts [4–6]. Indeed, the correlations of lectin binding to cells with active responses of the cells [7,8] and the impairment of stable-aggregate formation by inhibitors of distinct compounds of signaling pathways [9] lent credence to this concept. However, the effect of a signaling inhibitor may be non-specific and not essentially dependent on the lectin as the sole trigger molecule. It is thus necessary to add controls to this experimental approach testing the efficiency of inhibitors on cells exposed to different lectins known to induce disparate effects. As suitable models the lectins from *Canavalia ensiformis* (Con A) and *Solanum tuberosum* (STA) have been chosen, because they are sufficiently different in their signal-inducing capacity despite their common ability to induce sugar-inhibiting cell aggregation [10]. In particular, STA in contrast to Con A fails to induce an increase of cytosolic Ca^{2+} availability in rat thymocytes [11]. The aims of this study were to assess quantitatively the stability of thymocyte aggregates, whose formation is induced by the crosslinking capacities of STA and Con A, in the presence of haptenic sugars and to pinpoint any indication of a non-specific impact of signaling inhibitors on basal cell activities or the sugar-binding activity of the two lectins. The results obtained in the two systems provided evidence that the formation of HSR intercellular contacts was an inherent property of the individual lectin without any notable effect of the inhibitors on lectin binding to a glycoligand.

2. Materials and methods

Nordihydroguaiaretic acid (NDGA), α-methyl-D-mannopyranoside (α-MM), trifluoperazine (TFP), and *N*-ethylmaleimide (NEM) were obtained from Sigma (Deisenhofen, Germany); tricyclodecan-9-yl-xanthogenate potassium salt (D609) and 3-{2-[4-(*bis*-(4-fluorophenyl)-methylene]-1-piperidinyl)ethyl}-2,3-dihydro-2-thioxo-4(1*H*)quinazolinone (diacylglycerol kinase inhibitor II) were from Calbiochem (Bad Soden, Germany); dimethyl sulfoxide, emodin and *N*-acetyl-D-glucosamine (GlcNAc) were from Roth (Karlsruhe, Germany); chitin hydrolysate was from Vector (Burlingame, CA, USA); *Solanum tuberosum* agglutinin was from Lektinotest (Lvov, Ukraine); horseradish peroxidase was from Reanal (Budapest, Hungary). Con A was isolated from beans of *Canavalia ensiformis* by affinity chromatography using a horseradish peroxidase-Sepharose 4B matrix exposing mannose-rich core glycans [12] as affinity ligands [13].

Lymphoid cells were isolated from thymus glands of white non-inbred rats, as described [8]. Thymocytes were suspended in phosphate-buffered saline (PBS), pH 7.3, containing 10 mM Na_2HPO_4/KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2 \cdot 6H_2O$ and 5 mM D-glucose.

Extent of cell aggregation and aggregate dissociation were measured using the computerized aggregometer AP2110 from SOLAR (Minsk, Belarus). Rat thymocytes (5×10^6 cells/ml) were incubated with or without inhibitors for 2 min at 37°C and then a lectin was added to induce aggregation and finally after 3–4 min the haptenic sugar was added to dissociate sensitive lectin-cell complexes. The aggregation rate was calculated as percentage of V/V_o (V_o is the rate of cell aggregation in controls and V is the rate of cell aggregation in the

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Abbreviations: Con A, concanavalin A; D609, tricyclodecan-9-yl-xanthogenate potassium salt; GlcNAc, *N*-acetyl-D-glucosamine; HSR, haptenic-sugar-resistant; α-MM, α-methyl-D-mannopyranoside; NDGA, nordihydroguaiaretic acid; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; STA, *Solanum tuberosum* agglutinin; TFP, trifluoperazine

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presence of an inhibitor). The magnitude of the disaggregation reaction was calculated according to the equation:

$$\alpha = (I/I_{max})/(I_o/I_{max,o}) \times 100\%$$

where I and I_o refer to the final extent of light transmission of cell suspension after the addition of haptenic sugar in the presence and in the absence of an inhibitor; I_{max} and $I_{max,o}$ refer to the final level of light transmission of the suspension in the absence of haptenic sugar with and without an inhibitor, respectively.

The precipitation of Con A/horseradish peroxidase complexes was measured spectrophotometrically by recording of changes in the light transmission of samples at 540 nm, as described [14].

3. Results and discussion

STA and Con A mediated aggregation of rat thymocytes which was blocked by chitin hydrolysate (mixture of GlcNAc and its β1,4-linked di- to tetramers) and α-MM, respectively (Figs. 1 and 2). Contact formation was not sufficient to effect post-binding processes leading to firm, sugar-independent adhesion. Indeed, STA-dependent aggregates were completely dissociated by chitin hydrolysate (0.3%) or GlcNAc (0.5 M), whereas about 70% of Con A-induced aggregates maintained their stability at a concentration of α-MM of 6 mM and higher (Fig. 3). Evidently, the nature of the lectin and its ligand(s) appears to be an important property for the formation of additional HSR intercellular contacts, corroborating previous observations for the mannoside-specific fimbriae of *E. coli* [4] and the galactoside-specific mistletoe lectin [5,6,9].

Since Con A and STA differ in their effector properties with respect to the stimulation of intracellular signaling reactions in rat thymocytes [8], the influence of the presence of inhibitors of distinct signaling pathways on the dissociation response of cell aggregates was studied. The set of substances used included the recently proved adhesion inhibitors with respect to the galactoside-specific mistletoe lectin [9]. It should be noted that all tested inhibitors reduced the rate of aggregation that could be attributed to an involvement of metabolic pathways in the cell aggregation reaction. In this series of experiments we employed concentrations of haptenic sugars which in controls prevented the aggregation reaction without a pronounced disaggregation. It was found that inhibitors of $p56^{lck}$ tyrosine kinase (emodin) and phosphatidylcholine-specific phospholipase C (D609) caused an enhancement of sugar-induced disaggregation of rat thymocytes exposed to both lectins, Con A-dependent responses being fairly sensitive to the action of inhibitors. A non-specific inhibitor of lipoxygenases and potent antioxidant (NDGA) also induced similar effects with respect to both STA- and Con A-mediated adhesion. At the same time, a specific inhibitory action on the formation of HSR intercellular contacts was displayed by the diacylglycerol kinase inhibitor II, the dual calmodulin antagonist/protein kinase C inhibitor (TFP) and the permeable sulfhydryl reagent (NEM): in the presence of these substances the magnitude of α-MM-induced disaggregation of Con A-formed cell aggregates was significantly higher ($P < 0.05$) than in controls and in the case of chitin hydrolysate-induced disaggregation of STA-cell complexes (Table 1).

To exclude any direct effect of the chemicals on Con A, the crosslinking between Con A and horseradish peroxidase containing mannose-rich oligosaccharides [12,14] was studied in a cell-free system. As illustrated in Fig. 4, complexes of Con A and horseradish peroxidase were completely dissociated by

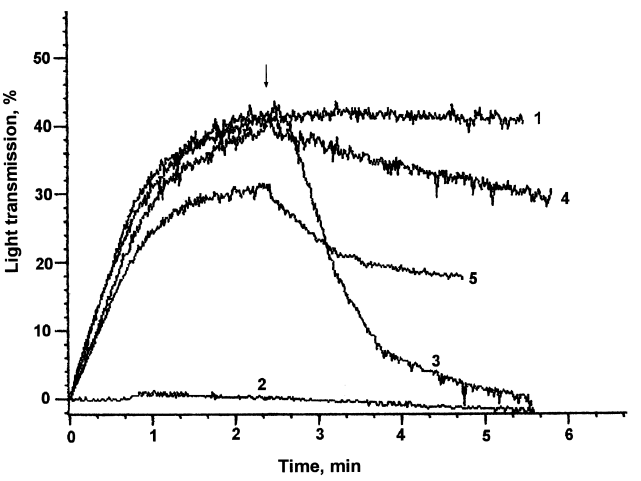

Fig. 1. Effects of chitin hydrolysate on the STA-induced aggregation of rat thymocytes (5×10^6 cells/ml). 1: normal kinetics of the lectin-induced aggregation of cells in the absence of chitin hydrolysate; 2: chitin hydrolysate (0.06%) was added to the cells prior to STA; 3: chitin hydrolysate (0.3%) was added at the moment indicated by the arrow; 4: chitin hydrolysate (0.06%) was added at the moment indicated by the arrow; 5: cell aggregation and disaggregation as in 4, but in the presence of emodin (10 μM). STA was used at the concentration of 100 μg/ml. Ordinate: light transmission, %.

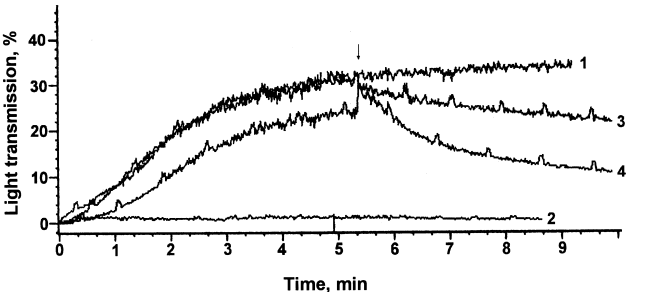

Fig. 2. Effects of α-MM on the Con A-induced aggregation of rat thymocytes (5×10^6 cells/ml). 1: normal kinetics of the lectin-induced aggregation of the cells in the absence of α-MM; 2: α-MM (2.4 mM) was added to the cells prior to Con A; 3: α-MM (2.4 mM) was added at the moment indicated by the arrow; 4: cell aggregation and disaggregation as in 3, but in the presence of D609 (50 μM). Con A was used at the concentration of 60 μg/ml. Ordinate: light transmission, %.

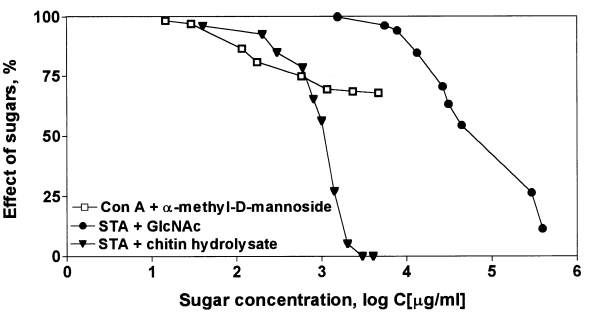

Fig. 3. Effects of different concentrations of haptenic sugar on the disaggregation of thymocyte aggregates formed by Con A (60 μg/ml) or STA (100 μg/ml). The concentration of the cells was 5×10^6 cells/ml. Ordinate: the extent of light transmission of the cell suspension (in %) relative to that of the control experiments in the absence of lectin-specific inhibitor.

Table 1
Effect of metabolic inhibitors on lectin-induced aggregation of rat thymocytes and haptenic-sugar-induced dissociation of cell aggregates

Substance (concentration)		Target of action	Con A, 60 μg/ml		STA, 100 μg/ml		
			V/V_o	α (α-MM)	V/V_o	α (chitin hydrolysate)	α (GlcNAc)
Diacylglycerol kinase inhibitor	10 μM	Diacylglycerol kinase in platelets	62.5 ± 11.8*	63.1 ± 6.1**	81.6 ± 4.9**	86.7 ± 11.0	88.2 ± 2.2
Nordihydroguaiaretic acid	50 μM	Lipoxygenases	69.2 ± 10.0*	73.0 ± 7.2*	70.3 ± 16.9*	75.8 ± 7.4*	87.3 ± 12.2
Trifluoperazine	10 μM	Calmodulin antagonist; protein kinase C	55.7 ± 6.7*	79.3 ± 4.2*	88.8 ± 4.3*	92.7 ± 1.4	98.4 ± 2.3
	50 μM		ND	ND	74.8 ± 10.6*	90.5 ± 5.1	93.1 ± 2.1
Emodin	5 μM	$p56^{lck}$ tyrosine kinase	80.2 ± 8.8*	69.4 ± 7.4*	84.1 ± 8.3*	79.7 ± 10.5*	85.1 ± 9.7
	10 μM		71.0 ± 9.7*	47.4 ± 19.2*	78.8 ± 4.2***	77.3 ± 6.9*	80.0 ± 6.2*
D609	10 μM	Phosphatidylcholine-specific phospholipase C	79.3 ± 8.7	67.6 ± 3.4**	89.7 ± 6.4*	95.2 ± 4.3	97.3 ± 2.3
	50 μM		70.8 ± 8.7*	41.5 ± 8.4**	78.4 ± 9.2*	79.7 ± 6.7*	85.6 ± 4.3
N-Ethylmaleimide	50 μM	Sulfhydryl alkylating reagent	66.5 ± 0.5**	57.1 ± 6.8*	89.7 ± 9.5*	92.6 ± 6.5	94.4 ± 1.7

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; the extent of aggregation and aggregate dissociation in control experiments without the use of any inhibitor was set at 100%. Sugar compounds were used at concentrations of 2.4 mM (α-MM), 0.06% (chitin hydrolysate), and 100 mM (GlcNAc). ND: not determined.

α-MM applied at the relatively high concentration of 7.5 mM. The tested inhibitors (emodin, diacylglycerol kinase inhibitor II, D609, NEM, and NDGA) at concentrations in the range of 25–50 mM failed to affect either the rate of precipitation reaction or the rate of precipitate dissociation. It should be noted that inhibitors did not affect the stability of precipitates even in the presence of low concentrations of α-MM not sufficient to completely block binding. The data thus raised no evidence for any effect of the substances on the lectin's capacity to form sugar-dependent complexes.

In conclusion, the obtained results intimate that the stability of plant lectin-induced cell aggregates is dependent on post-binding signaling which is characteristic of the individual type of lectin. Despite the similar aggregating activities of STA and Con A HSR contacts between rat thymocytes were formed only by Con A. This lectin, in contrast to STA, induces for example an increase in cytoplasmic Ca^{2+} availability in rat thymocytes [8], serving as a trigger with impact on other cell adhesion systems on the surface of different cells [15]. From the point of view of trans-membrane signaling mechanisms cell binding can be separated into active and inert complexes [16]. While the establishment of lectin-glycoligand complexes with a constant stoichiometry is considered to be pivotal for a signaling response [17], the actual complex constituents will determine the nature of subsequent reactions. Con A clearly fulfills this criterion with its multitude of triggered responses, e.g. in lymphoid cells an enhancement of mobility of phosphatidylcholine chains [18] and their deacylation [19] as well as the oxidation of sulfhydryl groups localized on the surface of the cell membrane [20]. As attested by this study, the application of target-selective inhibitors is conducive to delineating the contribution of individual signaling pathways to the measurable parameter, i.e. HSR aggregate formation. Signaling reactions associated with establishment of such stable intercellular contacts are indicated as being sensitive to blocking diacylglycerol kinase and protein kinase C and/or calmodulin activities, and perturbing the intracellular pool of thiols. It should be noted that the nature of the HSR contacts has remained unclear and only the application of monoclonal antibodies to the known adhesion molecules (selectins, integrins, adhesins of the Ig superfamily, and others) permits identification of the actual participants of the adhesion reaction. Obviously, the binding of STA to glycocomponents of membranes of rat thymocytes can only form bridges between cells without ensuing processes to initiate additional protein-protein (sugar) recognition. Lectin specificity on the level of oligosaccharides and topological arrangements of the complexes will govern the reactions for each cell type. This aspect deserves special attention for delineating the action of endogenous lectins in normal or pathological processes [21,22]. Hereby, the sugar code presentation on the cell surface is assumed to be a versatile hardware of dynamic aspects of cell sociology.

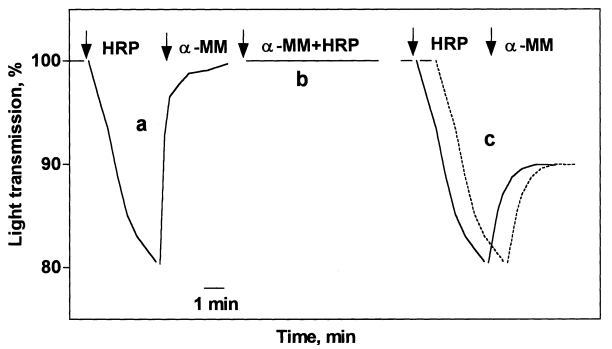

Fig. 4. Extent of precipitation of complexes between horseradish peroxidase (100 μg/ml) and Con A (75 μg/ml) in PBS, pH 7.3, measured by the changes of light transmission at 540 nm of the solution containing lectin and lectin-binding glycoprotein. a: Kinetics of Con A and horseradish peroxidase precipitation (decrease in light transmission) and dissociation of the detectable complexes by α-MM (7.5 mM) (increase of light transmission); b: blocking of complex formation by α-MM (1 mM); c: kinetic traces of precipitation of crosslinked lectin-glycoprotein complexes and their dissociation by α-MM (1 mM) in the absence (solid line) and in the presence (dotted line) of diacylglycerol kinase inhibitor II (50 μM), TFP (50 μM), NDGA (50 μM), emodin (25 μM), D609 (50 μM), or NEM (50 μM). The arrows indicate the moments of addition of horseradish peroxidase (HRP) and α-MM to the Con A-containing solution.

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