CHOLERA TOXIN INDUCED REDISTRIBUTION OF SIALOGLYCOLIPID RECEPTOR AT THE LYMPHOCYTE MEMBRANE

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1. Introduction

The interaction of polyvalent ligands, such as antibodies or lectins with cell surface structures of living lymphocytes or other cell types, often leads to a redistribution of plasma membrane constituents into clusters, patches and caps. The redistribution process is not fully understood but it is a metabolically dependent active process, possibly involving the activity of contractile microfilaments [1] and microtubules [2,6]. It appears likely that receptors of the membrane may partially be immobilized by cross linkages with the ligands [3]. As a consequence, the complexes formed become concentrated in the tail position of the cell, as the free membrane components flow forward as a part of the normal process of cell movement [4]. However, the inhibition of the Con A-induced cocapping of unrelated membrane receptors, e.g. by colchicine, points to an additional involvement of colchicine-sensitive microstructures. Following cap formation, the ligand–receptor complexes can be taken up by the cell through pinocytosis and/or be shed into the medium [5]. It is unclear which of those phenomena may perhaps be an important factor for possible triggering of the cell [4,6]. Ligand-induced redistribution has been demonstrated with antibodies directed against a variety of protein or carbohydrate [7] antigens located at the plasma membrane (immunoglobulins, θ, TL, H-2, HLA) and also for various phytomitogens, i.e. lectins (wheat germ phythemagglutinin, concanavalin A, pokeweed) and endotoxins, (for review see [1]).

Since membrane receptors which undergo capping have been envisioned as mobile units consisting of single or grouped protein molecules floating and diffusing in the plane of a fluid bilayer of lipid, it was of interest to see whether lipid also could directly take part in the capping phenomenon.

The glycolipid ganglioside G_{Gtet}l* which occurs localised in the plasma membrane of mammalian cells is known to interact strongly and specifically with cholera toxin [8,9]. Using fluoresceine-labelled cholera toxin, Craig and Cuatrecasas [10] have demonstrated a redistribution and cap formation on the surface of lymphocytes which was temperature-dependent and sensitive for metabolic inhibitors. This observation could be confirmed very recently by Revesz and Greaves [11], using fluoresceine-conjugated antibodies against cholera toxin. However, to prove unequivocally the lipid nature of the membrane receptor molecules, a more direct approach was chosen, using a fluorescent-labelled synthetic sialoglycolipid, in short: 'DANSyl-gangliosidoïde'. (fig.1). This lipid could be incorporated into the plasma membrane and reacted specifically with cholera toxin. After the fluorescent-labelled sialoglycolipid was bound to the cell membrane of lymphocytes, redistribution of the fluorescence occured and caps were formed upon reaction with the cholera toxin.

**Abbreviations:* Ganglioside G_{Gtet}l = Gal $\beta\rightarrow$3GalNAc$\beta\rightarrow$ 4Gal (3$\leftarrow\alpha$NeuAc) $\beta\rightarrow$4Glc-Ceramide PIS 04 = polyionic isotonic solution [20]. DANSyl = 5-dimethylamino-naphthalene-1-sulfonyl-.

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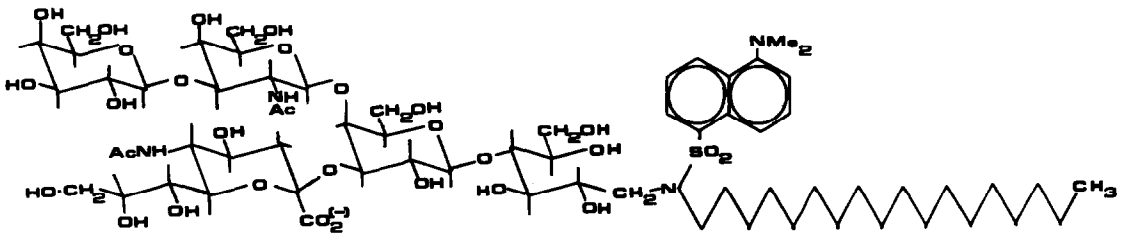

Fig.1. DANSyl-fluorescent-labelled cholera toxin-receptor sialo-glycolipid (Galβ→3GalNAcβ→4Gal (3←αNeuAc) β→4[1-desoxy-1-N-DANSyl-octadecylamino-]sorbitol (DANSyl-gangliosidoïde).

2. Materials and methods

2.1. *Synthesis of DANSyl-gangliosidoïde*

Ganglioside $G_{Gtet}l$ was isolated as described earlier [12]. The free sialo-oligosaccharide moiety of this ganglioside was prepared [13] and reductaminated in the presence of octadecylamine to yield Galβ1→3 GalNAcβ1→4Gal(3←2αNeuAc) β1→4[1-desoxy-1-octadecylamino-sorbitol] [14]. Following the method of Gray [15], this sialo-glycolipid was N-DANSylated to the DANSyl-gangliosidoïde shown in fig.1. The final product was purified by preparative thin layer chromatography on silica gel (E. Merck) with chloroform:methanol:water (65:35:8) as solvent system. The DANSyl-gangliosidoïde migrated on silica gel F254 (Rapid Fertigplatten Woelm) using the above system with R= 1.08 relative to ganglioside $G_{Gtet}l$.

2.2. *Preparation of the lymphocytes*

Lymphocytes were isolated from human peripheral blood by preincubation on glass bead columns and gradient centrifugation in a modification of Boyum's methods [16]. A part of the lymphocytes was pretreated with 0.25% trypsin for 30 min at 20°C. After excessive washings, various concentrations of untreated and pretreated lymphocytes in isotonic medium (PIS 04) were incubated with various amounts of DANSyl-gangliosidoïde for different time periods and at different temperatures. After washing 4 times, the cells were either immediately examined microscopically (viable under cover glass sealed with paraffin or dead on a smear) or the cells (sediment with 2×10^7 lymphocytes) were resuspended in and incubated with 0.1 ml cholera toxin protein I (0.1 nmol/ml, with or without cholchicine (10^{-6} mol/ml) for different times at different temperatures. Subsequently, the cells were examined microscopically in the same way as mentioned above.

2.3. *Microscopical examinations*

A Zeiss photomicroscope was used equipped with a HBO 200 light source, a Ploem type vertical illuminator and a III RS-condensor with an excitation filter UGI; reflector Fl 400 and barrier filter 41. For fluorescein fluorescence, the excitation filter G 455, 2 X KP 500, reflector FL 500 and barrier filter 50 were used. All microscopical examinations were performed with a magnification of 1250 times.

2.4. *Precipitation of cholera toxin-subunit-protein I with DANSyl-gangliosidoïde*

Cholera toxin-subunit protein I, equilibrated with sodium phosphate buffer, 0.1 M, 0.5 mM EDTA, pH 7.2, was adjusted to a concentration of 5 nmol/ml (M_R = 54.000). The DANSyl gangliosidoïde or ganglioside $G_{Gtet}l$, both at concentrations of 0.1 μmol/ml of the above-mentioned buffer were mixed with aliquots of the solution of protein I. Mixtures at molar ratios of 0.1:1, 1:1, 2:1, and 4:1 for the glycolipid and protein I complexes were prepared. Precipitate formation was visually estimated 20 h later and the inhibition of immunoprecipitates was evaluated by immunodiffusion against antiserum to protein I, essentially as described earlier [9].

3. Results

3.1. *Interaction of DANSyl-gangliosidoïde with protein I-subunit of cholera toxin*

The DANSyl-gangliosidoïde precipitated protein I.

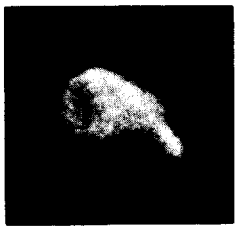

Fig.2. Capping of DANSyl-gangliosidoïde-treated lymphocyte induced by treatment with cholera toxin-subunit protein I.Ig receptors of the lymphocyte redistribute into the same cap.

However, differences in the formation of insoluble precipitates with protein I were observed for the DANSyl-sialoglycolipid as compared to ganglioside G_{Gtet}I. The DANSyl-gangliosidoïde precipitated protein I at a molar ratio as low as 1:1, whereas ganglioside G_{Gtet}I protein I complexes became insoluble only at a molar ratio of 4:1. However, as can be seen from fig.2, the DANSyl-gangliosidoïde as well as ganglioside G_{Gtet}I caused an inhibition of immunoprecipitation between protein I and its antiserum at molar ratios higher than 1:1. This differential behaviour is closely related to the observations made with gangliosidoïde and ganglioside cholera toxin complexes [9].

3.2. *Binding of DANSyl-gangliosidoïde to the membrane of lymphocytes*

The incorporation of DANSyl-gangliosidoïde into the membrane of lymphocytes and granulocytes was clearly temperature dependent. Addition of 1 or more nmol DANSyl-gangliosidoïde to 5×10^6 lymphocytes at 37°C resulted in a pronounced surface fluorescence of all the lymphocytes or contaminating granulocytes. However, the fluorescence intensity was variable. Lowering the incubation temperature to 20°C, 4°C or 0°C led to a significantly reduced fluorescence intensity and a decreased number of fluorescent-labelled lymphocytes. This effect could be observed in lymphocytes from all donors examined but appears to be quantitatively different between the single donors. The temperature dependence of the incorporation of DANSyl-gangliosidoïde points to the fact that this glycolipid is incorporated into the lipid bilayer proper of the outer cell membrane.

3.3. *Effect of cholera toxin subunit-protein I on lymphocytes labelled with DANSyl-gangliosidoïde (table 1)*

Subsequent incubation of DANSyl-gangliosidoïde-treated lymphocytes with cholera toxin protein I-subunit caused a time- and temperature-dependent redistribution of the membrane bound fluorescence. After 10 minutes incubation at 37°C, cap formation was observed in a few cells. After 20 min of incubation, the number of cap forming cell increased significantly compared to cells incubated similarly at 4°. Caps could be located distinctly in the uropod region of the lymphocytes.

Lymphocytes treated with DANSyl-gangliosidoïde but without any post-treatment of cholera toxin-protein I formed caps too. However, the percentage of those cap-forming cells was temperature independent and was much lower than after treatment with protein I at 4°C (table 1).

3.4. *Influence of colchicine*

Addition of colchicine (10^{-6} M) to the incubation medium reduced the number of protein I induced cap forming DANSyl-gangliosidoïde-treated lymphocytes in all cases (table 1).

3.5. *Effect of trypsin (table 1)*

Pretreatment of lymphocytes with trypsin appears to have no effect on the uptake of DANSyl-gangliosidoïde by the membrane of lymphocytes or granulocytes, as could be judged from the fluorescence intensity of the preparations. However, cap formation induced by cholera toxin protein I was markedly reduced by a pretreatment of the cells with the protease.

3.6. *Cocapping of lymphocyte anti-immunoglobulin receptors*

In order to study a possible cocapping of the immunoglobulin G receptors present on the surface of peripheral lymphocytes, white blood cells treated differently in the way described above, were subsequently incubated with fluoresceine-conjugated anti-human-Ig polyvalent antibodies of the rabbit (Op-Nr. F642, Behring Werke, Marburg) for 30 min at 20°C. The presence of Ig-receptors was observed. Ig-receptors redistributed into caps after DANSyl-gangliosidoïde pretreatment as well as after additional treat-

Table 1
Cap formation of DANSyl gangliosidoïde cholera toxin protein I complexes on the surface of human lymphocytes

Incub. Temp.	10^{-9} mol/[a] ml	% of cells with cap formation			
				Trypsin	
		Colchicine[c]			Colchicine[c]
4°C	1, control[b]	2		2	
	1	2	1	3	2
	10	11	6	7	2
37°C	1, control[b]	3		3	
	1	9	3	6	2
	10	25	11	5	7

[a] Concentration of DANSyl gangliosidoïde (0.1 ml) per 1 × 10^7 lymphocytes (sediment). Incubation for 15 min. After 4 washes subsequently, 0.01 nmol cholera toxin protein I per 1 × 10^7 lymphocytes for 30 min.
[b] Control, incubation with 1 × 10^{-9} mol/1 × 10^7 cells/0.1 ml DANSyl gangliosidoïde but without subsequent incubation with cholera toxin protein I.
[c] Simultaneous incubation with colchicine (1 × 10^{-7} mol/1 × 10^7 cells/0.1 ml).
[d] Pretreatment of lymphocytes with 0.25% trypsin, 30 min., 20°C.

ment with cholera toxin protein subunit I. However, in the case of glycolipid capping, Ig-receptors always moved into the same cell pole as the DANSyl-ganglioside-protein I complex.

4. Discussion

The ganglioside-membrane-receptor binding protein subunit of cholera toxin, i.e. protein I instead of the intact toxin itself, was chosen for this investigation in order to avoid any interference arising from an activation of the cell's adenylate cyclase system by the toxin subunit protein II [17]. The synthetic gangliosidoïde which carries a DANSyl-fluorescent label, binds to protein I of cholera toxin strongly and specifically as it is known for the natural toxin receptor, the ganglioside $G_{Gtet}l$. Moreover it could be demonstrated that DANSyl-gangliosidoïde binds to the cell membrane, perhaps in a similar fashion as has already been shown for other gangliosides [18,19]. Since the binding is temperature dependent, it can be argued that the incorporation of the DANSyl-gangliosidoïde took place into the lipid bilayer of the membrane. In vitro addition of the cholera toxin-subunit protein I to DANSyl-gangliosidoïde leads to the formation of a precipitate. At the lymphocyte surface membrane this interaction results in a redistribution of the fluorescent lipid with formation of caps. This shows that the incorporated DANSyl-gangliosidoïde is mobile laterally in the plane of the surface membrane. The ligand-induced mobility is dependent on the temperature. It is also sensitive to colchicine, a fact already shown for membrane gangliosides by indirect methods [10]. However, in contrast to the results with indirect methods [12], ligand-induced mobility of DANSyl-gangliosidoïde is influenced by trypsin pretreatment. Moreover, DANSyl-gangliosidoïde-cholera toxin-protein I complexes redistribute into the same cap as the lymphocyte anti-immunoglobulin receptors. These findings strongly suggest the possibility that lipids do not move independently but are influenced by or comigrate with other membrane components.

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