Volume 264, number 2, 249–252 FEBS 08443 May 1990

Effect of gangliosides on binding, internalization and cytotoxic activity of ricin

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Received 28 March 1990

The only gangliosides in Burkitts' lymphoma EB-3 cells is GM3. Treatment of Burkitts' lymphoma EB-3 cells with ganglosides GM1 or GM3 results in their binding to and partial incorporation into the cell membrane. About 25% of cell-associated ganglioside GM1 can interact with the ricin. However, such an increase in the number of binding sites does not enhance but rather decreases the cytotoxic effect of ricin. A similar protective effect was observed when the cells were pretreated with ganglioside GM3. In contrast, the increase in ricin binding sites caused by pretreatment of the cells with neuraminidase was accompanied by increase in ricin cytotoxicity. These differences may be related to observed differences in the rate of ricin-endocytosis by native and ganglioside-treated cells.

Ricin; Receptor; Ganglioside

1. INTRODUCTION

The plant toxin ricin is a 62 kDa glycoprotein consisting of two disulfide-bonded subunits [2]. The A chain (30 kDa) catalytically inactivates eukaryotic ribosomes [3]. The B chain (32 kDa) is a lectin that binds to galactose or *N*-acetylgalactosamine residues on the cell surface [4]. It is not clear, however, whether all such glycoconjugate molecules can serve as true receptors of the toxin (in the sense that they promote transfer of the toxin into the cytosol) or if only a subpopulation of the ricin binding sites is able to induce entry of the toxin. We have recently shown that Burkitts' lymphoma EB-3 cells contain two types of ricin binding sites [5]. To elucidate the nature of ricin receptors in Burkitts' lymphoma cells, we determined in the present study the binding of ricin to and its cytotoxic action on native cells, neuraminidase-treated cells and cells loaded with either ganglioside GM1 which contains a terminal galactose and can effectively bind ricin in model systems [6,16] or with GM3 having a terminal sialic acid residue.

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Abbreviations: BSA, bovine serum albumin; DMPC, 1,2-dimiristoylphosphatidylcholine; ASM, anthrylvinyl-labeled sphingomyelin (*N*-12-(9-anthryl)-*trans*-dodecenoylsphingosine-1-phosphocholine). Abbreviations of gangliosides follow the nomenclature of [1]: GM1, Gal(β-3)GalNAc(β1-4)NeuAc(α2-3)Gal(β1-4)Gal(β1-1)Cer; GM3, NeuAc(α2-3Gal(β1-4)Glc(β1-1)Cer

2. MATERIALS AND METHODS

Burkitts' lymphoma EB-3 cells were cultured in DMEM (Flow Labs) supplemented with 10% FCS (Flow Labs), 2 mM glutamine and canamycin (100 μg/ml) at 37°C in a humidified atmosphere with 5% CO_2 in air.

Ricin and its constituent polypeptide chains were isolated by the method described in [7,8]. Labeling of ricin B-chain with iodine-125 was performed according to [9] in the presence of 50 mM lactose.

Rabbits were immunized with ricin B-chain and antibodies were isolated using B-chain-Sepharose.

Binding of [^{125}I]B-chain and anti-B-chain antibodies was prepared as described in [10].

GM1, GM3 and labeled [^{3}H]GM1, [^{3}H]GM3 with a specific activity of 400 and 800 mCi/mM, respectively, were kindly provided by Dr N.V. Prokasova. The concentration of gangliosides in the incubation mixture containing 10^7 cells/ml was 200 μM. Uptake of gangliosides was determined after repeated washing of the cells.

The fluorescent probe ASM was kindly provided by Dr J.G. Molotkovsky. Fluorescent labeling and fluorescence anisotropy measurement were prepared as described by Manevich et al. [11].

Treatment of cells with neuraminidase. Washed lymphoma cells (10^7 cells/ml) were treated with neuraminidase from *Vibrio cholera* (Sigma) (50 U/ml) in Hanks' solution containing 1 mg/ml BSA (Calbiochem), for 1 h at 37°C [11].

The cytotoxic effect of ricin was determined by measuring the inhibition of [^{14}C]leucine incorporation into EB-3 cells caused by the toxin [12].

Small unilamellar liposomes were prepared by evaporation in vacuo of DMPC intermixed with ganglioside GM1 (9:1 M/M) solution in chloroform. The mixture was then suspended in PBS containing 1 mM EDTA, and sonicated for 5 min in a nitrogen atmosphere (50 W, 22–28 kHz; Labline Inc) at 0°C. After sonication the suspen-

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00145793/90/$3.50

sion was kept at 28°C for 2 h. The main phase transition was registered by liposome turbidity measurement [14] at 350 nm using a Beckman DU-8 (USA) spectrophotometer.

3. RESULTS AND DISCUSSION

3.1. *Loading of Burkitts' lymphoma cells with gangliosides*

Preliminary experiments demonstrated that upon incubation of the cells with increasing amounts of gangliosides in the 8–200 μM range, maximal binding was achieved after 20–30 min and the amount of cell-associated ganglioside increased linearly with the amount of ganglioside added [9].

After incubation of Burkitts' lymphoma cells the trypsin-resistant cell-associated ganglioside GM1 corresponded to about 40% of the total cell-associated ganglioside [9]. Earlier it was shown that incubation of gangliosides with mouse fibroblasts was not accompanied by ganglioside endocytosis and that all cell-associated gangliosides remained on the cell surface [15]. If this holds true also for EB-3 cells, their incubation with GM1 should result in the appearance of about 10^7 additional ricin binding sites' on the surface of each cell.

3.2. *Binding of ricin B-chain to native and modified EB-3 cells*

The binding of ^{125}I-ricin B-chain to Burkitts' lymphoma cells was determined by radioligand measurements. The number of high-affinity (K_A 10^{10} M^{-1}) and low affinity (K_A 10^8 M^{-1}) binding sites comprised 10^4 per cell and 10^6 per cell, respectively (Fig. 1A).

Neuraminidase treatment of EB-3 cells led to appearance of additional terminal galactose residues and resulted in a 12–18-fold increase of B-chain binding (Table I and Fig. 1B). Comparison of these data with the number of lipid-bound terminal galactoses appearing on the cell surface after neuraminidase treatment [9] demonstrated that the larger part (80%) of the B-chain-binding sites' increase must be due to protein-bound galactoses, which before neuraminidase treatment were masked.

Loading of the cells with exogenous GM1 led to a 2–3-fold increase of ricin B-chain binding, whereas loading of the cells with GM3 had no effect on the binding of B-chain (Table I).

3.3. *Effect of cell treatment on ricin endocytosis rate and cytotoxic action*

The 12–18-fold increase in ^{125}I-ricin B-chain binding following neuraminidase treatment of Burkitts' lymphoma cells (Table I) was accompanied by a marked increase in ricin cytotoxicity (Fig. 2). Contrary to this, the ricin-sensitivity of the ganglioside-loaded cells was about 2 times lower than that of control cells. A similar cytoprotective effect was observed upon loading of the

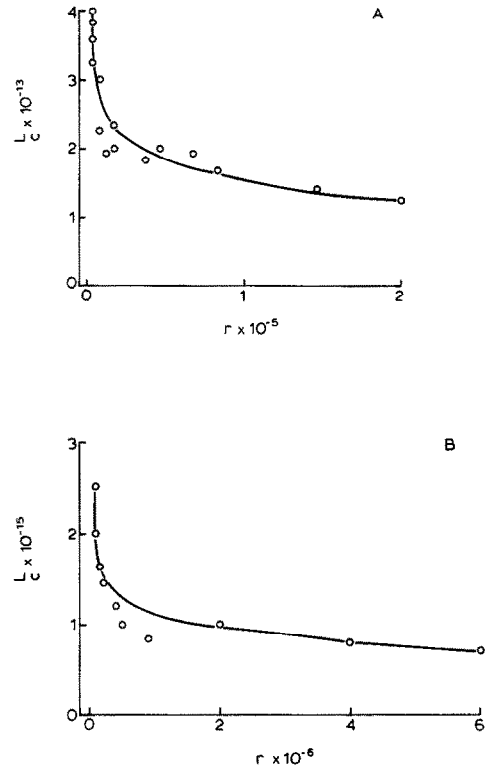

Fig. 1. Binding of the ricin B-chain to native (A) and neuraminidase-treated (B) Burkitts' lymphoma cells. Data are presented in Scatchard coordinates, where r is the number of ricin B-chain molecules bound to one cell, and c the concentration of non-bound ricin B-chain in mol/l. Values are averages of quadruplicate incubations from a single experiment.

cells with ganglioside GM3. Loading of the cells with ganglioside GM1 resulted in a decrease of the endocytosis rate: after 20 min incubation of ricin-treated GM1-loaded cells the binding of anti-ricin B-chain-antibodies was two times higher than in ricin-treated control cells, but after 60 min incubation about 90% of the cell-associated ricin became inaccessible to anti-ricin B-chain-antibodies both in GM1-loaded cells and in control cells (Table II). A similar decrease in the rate of ricin endocytosis after treatment of cells with con-

Table I

Binding of ^{125}I-ricin B-chain to Burkitts' lymphoma cells pretreated with neuraminidase or gangliosides

Concentration of ^{125}I-ricin B-chain (10^3 cpm·ml^{-1}·min^{-1})	Cell-bound ^{125}I-ricin B-chain (10^3 cpm·min^{-1})			
	Buffer	Cells treated with		
		Neuramin-idase	GM1	GM3
16	2.0	25	5.0	2.2
70	5.7	93	13.5	5.2
250	8.6	–	26.8	8.8

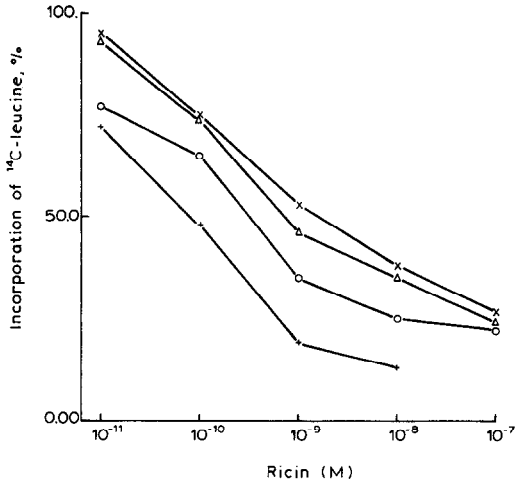

Fig. 2. Cytotoxic action of ricin on native EB-3 cells (○), cells pretreated with neuraminidase (+), cells loaded with ganglioside GM1 (▲) and ganglioside GM3 (×).

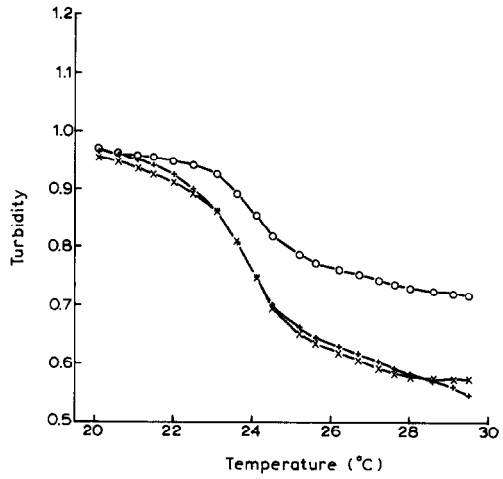

Fig. 3. Influence of ricin (+) and ricin B-chain (×) on the phase transition of DMPC-liposomes containing 2 M% of GM1 at pH 7.5, registered by turbidity measurement; (○) liposomes without any protein.

canavalin A, was ascribed to an increase in the rigidity of the plasma membrane effected by the lectin [17].

3.4. *Effect of ricin binding on cell membranes and liposomes*

Earlier the binding of ricin to Burkitts' lymphoma cells was studied using a fluorescence method based on measurement of the ligand-induced changes in the fluorescence anisotropy of the concentration-dependent, saturable fashion [5]. In the present study loading of ASM-labeled EB-3 cells with ganglioside GM1 resulted in some decrease of the ASM-fluorescence anisotropy (approximately 8%) (and hence probably in a somewhat more fluid plasma membrane) but upon addition of ricin B-chain (10^6 molecules per cell) no additional change in the fluorescence polarization (as compared to ASM-labeled control cells) was observed.

As can be seen from Fig. 3, ricin and B-chain increased the abruptness of the phase transition of DMPC-liposomes containing 2 M% GM1 indicating an increase in cooperativity of the phase transition. With increasing GM1 concentration the phase transition of DMPC in the presence of ricin B-chain was broadened (Fig. 4) testifying to a loss in cooperativity. This phenomenon may be explained in terms of ganglioside clustering induced by the ricin B-chain.

As noted above, loading of EB-3 cells with GM1 by contrast, increases the apparent fluidity of the plasma membrane. However, it is not excluded that in the presence of ricin gangliosides cluster around glycoprotein receptors of ricin, forming rigid microdomains impeding endocytosis.

In conclusion, the combined data of the present study show that GM1 and, probably related glycolipids, which have terminal galactoses, are not the true receptors of ricin. Probably the true ricin receptors belong to the relatively small population of molecules binding the toxin with higher affinity.

Table II

Binding of ^{125}I-labeled anti-ricin B-chain-antibodies to ricin-treated lymphoma cells

Time of incubation at 37°C before addition of antibodies to ricin-treated cells (min)	% of bound antibodies[a]	
	Native cells	GM1-treated cells
0	100	100
20	17	34
60	10	9

[a] % of bound antibodies = (binding of ^{125}I-antibodies after incubation at 37°C/binding of ^{125}I-antibodies at 0°C) × 100%

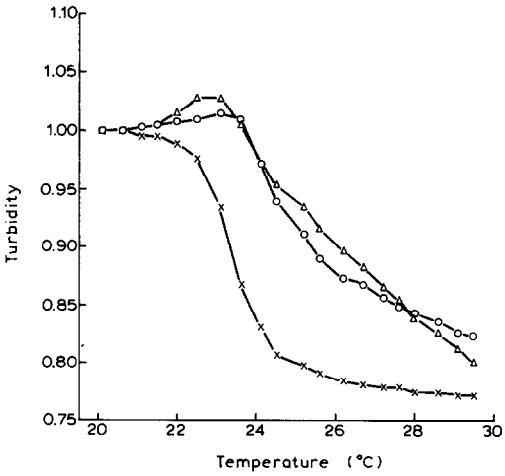

Fig. 4. Influence of GM1 on the phase transition of DMPC-liposomes preincubated with ricin B-chain at pH 7.5: (×) 2 M% GM1; (○) 4 M% GM1; (▲) 8 M% GM1.

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