THE USE OF CONCANAVALIN A IN THE STUDY OF THE DYNAMICS OF LYMPHOCYTE MEMBRANE GLYCANS

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1. Introduction

Concanavalin A (Con A) is a protein obtained from *Canavalia ensiformis* which specifically interacts with terminal α-D-mannopyranosyl, α-D-glucopyranosyl, and β-D-fructofuranosyl residues of polysaccharide chains [1]. The use of this plant lectin for the detection, and the quantitation of such cellular residues, has been previously described [2]. Moreover, Con-A induces blastic transformation of lymphocytes [3]. The lymphocyte–Con A interaction provides a good model for studying lymphocyte membrane movements and lymphocyte stimulation mechanisms. In this work, we report experiments performed on the rate of disappearance of Con A receptors from lymphocyte surface membranes and their regeneration kinetics.

2. Materials and methods

2.1. *Animals*

Animals were Sprague Dawley female rats five months old.

2.2. *Medium and cell suspensions*

Cervical lymph nodes were teased in Hank's balanced salt solution in which galactose replaced glucose. This was done in order to avoid inhibition by glucose of the Con A binding to the cell receptors. For the same reason, no serum was used at any stage of the present study. The cell suspension was washed in Hank's medium by three successive centrifugations (250 *g*; 7 min; 4°). Cells were then counted in an hemacytometer, and their viability was estimated by trypan blue exclusion.

2.3. *Estimation of the amount of membrane bound Con A.*

The binding of Con A on the surface of lymphocytes was estimated by a procedure which measures Con A by peroxidase activity [2]. To do this, 1 ml of medium containing 5×10^7 cells and 400 μg of Con A was incubated for 15 min at +4°. The cells were then washed at 4° by three successive centrifugations, and the pellet was resuspended and incubated for 15 min in 5 ml cold Hank's medium containing 50 μg/ml of peroxidase. After three more washings the cell pellet was suspendend in 2 ml of physiological saline buffer containing 0.1 M of α-methyl-D-mannoside, a competitive inhibitor of Con A binding, and incubated for 15 min at room temp. The cells were finally centrifuged, and the peroxidase released in the supernant was measured using H_2O_2 as substrate, and *O*-dianisidine as the chromogenic substance [4, 5]. The number of Con A molecules attached per cell was calculated from the number of peroxidase molecules bound per 10^6 cells, by referring to a standard peroxidase activity curve established with known concentrations of peroxidase.

2.4. *Disappearance of Con A molecules from lymphocyte membranes*

After treatment with Con A at 4° for 15 min, and three washings, the cells were suspended and incubated in Hank's medium adjusted to various temperatures. One ml samples were withdrawn at various intervals, added in 4 ml of 60 μg/ml peroxidase solution and

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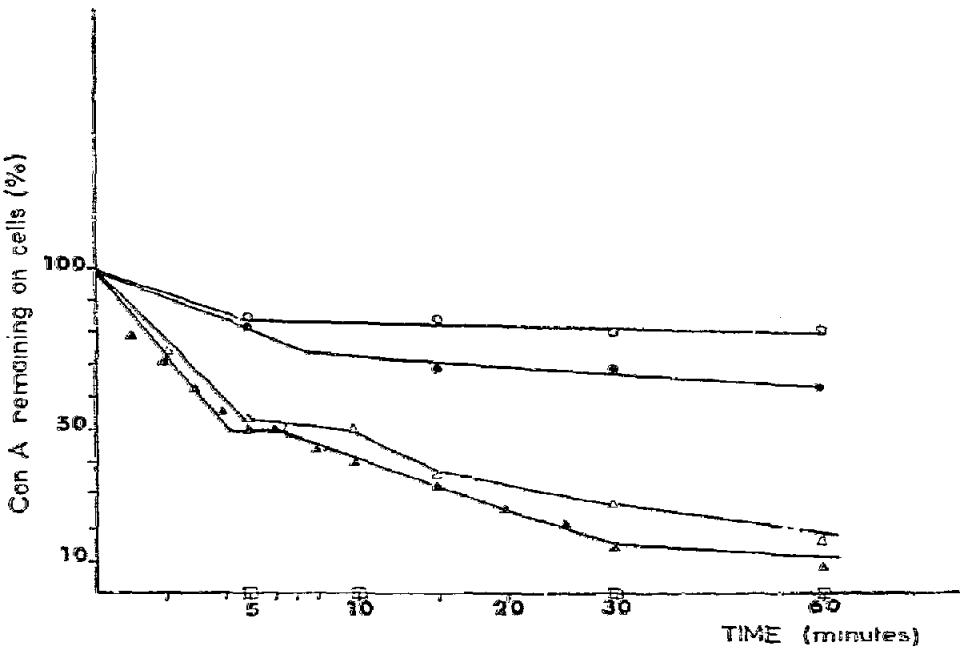

Fig. 1. Disappearance of Con A from cell surface as a function of time and temperature. (▲—▲—▲) Incubation of lymphocytes at 37°. (●—●—●) Incubation of lymphocytes at 24°. (○—○—○) Incubation of lymphocytes at 10°. (△—△—△) Incubation of thymocytes at 37°. (□—□—□) Cells incubated in the presence of α-methyl-D-mannoside (at all stages). Each point represents mean values of three different experiments. Standard error for each point = ± 10%.

then processed at described in sect. 2.3. For control purposes cells were treated in the same way, but α-methyl-D-mannoside was present during all working steps.

2.5. *Reappearance of Con A receptors on the lymphocyte membrane*

The Con A treated cells were washed, suspended in Hank's medium, and incubated at 37°. After varying times, three samples of 1 ml each were withdrawn. The first sample was incubated with peroxidase as described in sect. 2.2., in order to measure the Con A remaining on the cell surface. The second sample was treated first with 400 μg/ml of Con A and then washed before incubation in peroxidase. The third sample was treated with 0.1 M α-methyl-D-mannoside, in order to elute the Con A remaining on the cell surface, washed and was then treated as previously with Con A and peroxidase.

3. Results

Incubation of a constant number of cells (5×10^7 /ml) with varying concentrations of Con A showed that the amount of Con A fixed on the cells increased with concentration and reached a plateau at values higher than 400 μg/ml of Con A. In these conditions, each lymphocyte binds about 7×10^5 molecules of Con A.

Fig. 1 shows the disappearance of Con A from cell surface as a function of time and temperature. It can be seen that Con A disappears at two different rates and that this process is temperature dependent. As blastic transformation and antigenic modulation are dose dependent phenomena, it was of interest to check the relationship existing between quantity of Con A attached to the cells and its subsequent disappearance from the cell surface. The results obtained are shown in fig. 2. Variations in the slope of the

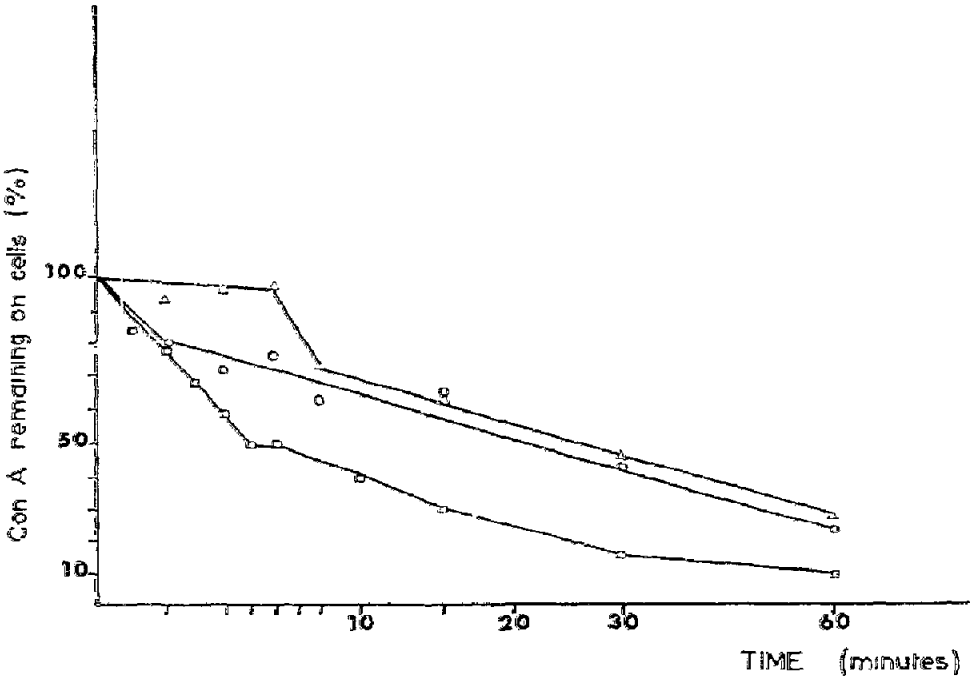

Fig. 2. Dose dependence of the disappearance of Con A from the cell surface at 37°. (□–□–□) 7×10^5 Con A molecules/cell. (○–○–○) 7×10^4 Con A molecules/cell. (△–△–△) 2×10^4 Con A molecules/cell. Cells are incubated for 15 min at 4° with 400 μg/ml, 50 μg/ml and 25 μg/ml of Con A, respectively. Each point represents mean values of three different experiments. Standard error for each point = ± 10%.

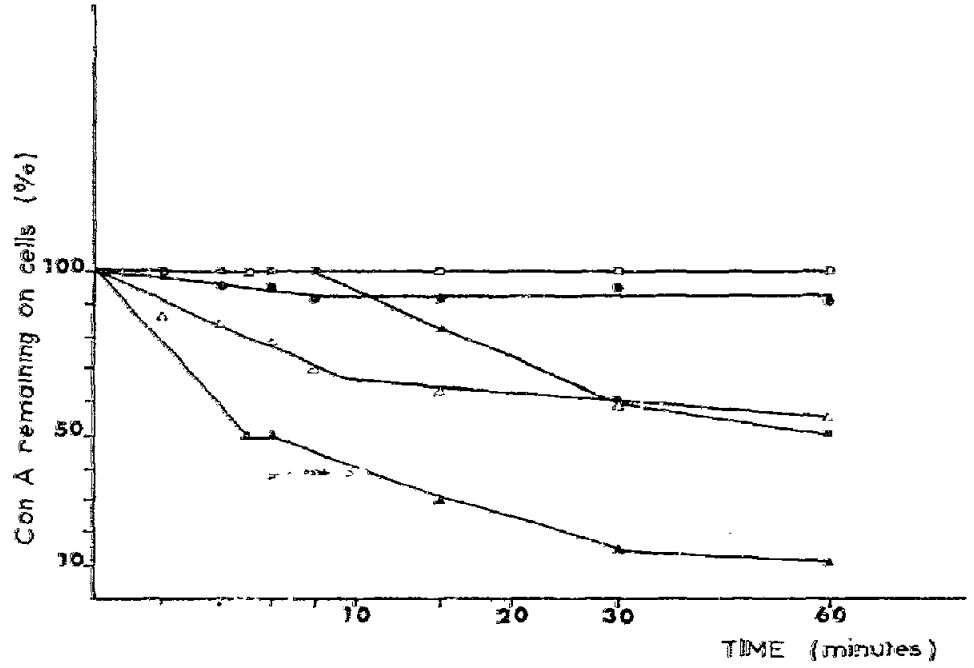

Fig. 3. Effect of various inhibitors on disappearance of Con A from cell surface. (□–□–□) Incubation of lymphocytes with DNP, 10^{-3} M at 24°. (●–●–●) Incubation of lymphocytes with 10 μg/ml cytochalasin B at 24°. (△–△–△) Incubation of lymphocytes with 10 μg/ml cytochalasin B at 37°. (■–■–■) Incubation of lymphocytes with ouabain 10^{-3} M at 37°. (▲–▲–▲) Disappearance of Con A without inhibitor. Each point represents mean values of three different experiments. Standard error for each point = ± 10%.

early phase were observed, but the slope of the late phase seemed not to be affected.

In order to determine if this apparently composite phenomenon was dependent upon energetic metabolism, (Na^+, K^+) ATPase, and membrane or microfilaments function, we tested the action of dinitrophenol (DNP) 10^{-4} M at 24°, ouabain 10^{-3} M at 37° and cytochalasin B (10 μg/ml or 50 μg/ml) at 37° and 24°. In these experiments, cells were preincubated for 30 min at 37° with the drug, before treatment with Con A. No cytotoxicity was observed.

Fig. 3 shows that disappearance of Con A from lymphocyte membrane is completely inhibited by DNP 10^{-4} M or by cytochalasin B at 24°. At 37°, cytochalasin B (10 μg/ml or 50 μg/ml) seems to slow down the first phase and to inhibit the second one. On the other hand ouabain inhibited Con A disappearance during the first minutes following incubation of the cells at 37° although the late and slower phase of the process was not affected. The effect of DNP was also checked at 37°, but the high cytotoxicity of this drug at this temperature makes the results doubtful.

Fig. 4 shows the reappearance of Con A receptors on the cell surface. It is evident that the number of receptors present at the surface of lymphocytes is increased when the cells are treated once with a saturating dose of Con A (400 μg/ml) then washed and treated again with Con A under the same conditions. The same increase in the number of Con A binding sites is observed when the lectin molecules adsorbed on the cells are first removed by α-methyl-D-mannoside before a second treatment of the cells with Con A. In other respects, the incubation of the cells for various time at 37° after the first treatment by Con A do not influence the number of membrane receptors available for new Con A molecules (fig. 4).

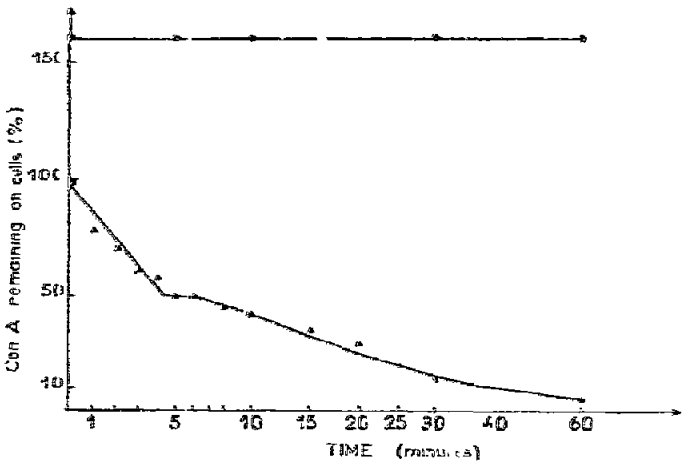

Fig. 4. Regeneration kinetics of Con A receptors. (▲–▲–▲) Disappearance of Con A from the cell surface (●–●–●) Detection of Con A receptors present at the surface of cells which have been submitted to the following treatment: incubation at 4° in Con A, washing; incubation at 37° for various times. Incubation in Con A at 4°. Each point represents mean values of three different experiments. Standard error for each point = ± 10%.

4. Discussion

Con A molecules disappear from lymphocyte membrane, following a two phase process: an early rapid phase and a slower late phase. The temperature dependence of this phenomenon suggest that fluidity of the membrane and/or energy are needed. Differential dependence of the early and late phases on the number of Con A molecules adsorbed per cell, suggests that Con A elimination from membrane, is a composite mechanism, including one system which is dependent on the cell surface concentration of Con A. Inhibition of the phenomenon by DNP seems to indicate that an energetic metabolism is required. Moreover, the kinetic pattern observed in the presence of ouabain suggests that rapid disappearance of Con A needs a stimulated (Na^+, K^+) ATPase. The action of cytochalasin B on the cells is not clear, but it appeared to act at the membrane level. Thus selective effect on the transport of small molecules [6] and inhibition of capping were reported to occur with this drug [7]. In our case cytochalasin B seems to act in the same way as a temperature decrease, but it is difficult to decide what is the actual target of this drug.

The regeneration kinetics obtained seems to indicate that a two step fixation of Con A on the membrane reveals more sites than a one step fixation under saturation conditions. Moreover, at 37° new Con A receptors seem to be present in the membrane, immediately after disappearance of Con A molecules. The first observation is puzzling, but it may be explained by a general conformational change of the glycan subunit assembly in the membrane, provoked by the fixation of Con A. The second observation may be explained by two hypotheses:

i) Only Con A is shed from cell surfaces either by pinocytosis or by elution.
ii) Con A is shed with the glycan of the membrane, and in this case the rapidity of new receptors reappearance requires the presence of preformed glycans ready to be inserted at the external face of the membrane. This preexistence of glycans may be explained by the model proposed by Singer and al. [8]. Work is now in progress to determine if Con A is eluted, pinocytosed, or both and if some membrane components are eluted simultaneously with Con A molecules.

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