ACTION OF CONCANAVALIN A ON THYMOCYTES STUDIED BY A FLUORESCENT PROBE *

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SUMMARY: The binding of 1-anilinonaphthalene-8-sulfonate (ANS), a fluorescent probe, to rat thymocytes treated with concanavalin A (con A) was investigated. Fluorescence intensity of bound ANS was greatly enhanced by prior incubation of the cells with con A at 37°C. The addition of α-methyl-D-glucoside, a specific inhibitor of the binding of con A to the cells, or treatment of the cells with con A at 0°C inhibited this fluorescence enhancement. The apparent dissociation constant of the binding of ANS to thymocytes was found decreased by the treatment with con A. Such an effect of con A was temperature-dependent: a transitional fluorescence enhancement occurred when the cells were treated with con A at about 20°C. Incubation of the cells with colchicine prior to the con A treatment abolished this effect of con A.

Mitogenic stimulation of lymphocytes by con A, one of mitogenic lectins, leads to the syntheses of proteins, RNA and DNA. Since these syntheses leading to mitosis of lymphocytes are believed to be triggered by the binding of con A to the cell surfaces (1), con A is a useful tool for investigating a possible linkage of surface events to the metabolic machinery of the cytoplasm and nucleus in cell proliferation. Although various metabolic changes associated with the stimulation by mitogens including con A have been reported (2-7), early events occurring in plasma membranes of lymphocytes after the binding of mitogens are still obscure. Aggregation of con A-receptors on the lymphocyte surface was reported to be necessary for mitogenic stimulations (8). Recently, Toyoshima and Osawa reported an increase in microviscosity of lymphocyte membranes following mitogenic stimulations (9). In this communication we report an increase in the binding of ANS to thymocytes treated with con A, suggesting a change in the properties of plasma membranes of lymphocytes shortly after the binding of the lectin.

MATERIALS AND METHODS

Thymocytes were obtained from the thymuses of 120-150 g Sprague-Dawley rats by mincing the tissues with a small scissors. The cells were washed and suspend-

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ed in 0.85% saline. More than 99% of the cells excluded 0.1% trypan blue. For measuring the binding of ANS to the cells, the cells were suspended in 2 ml of 0.05 M phosphate-buffered saline (PBS), pH 7.0, and were incubated with or without con A for given periods of time before adding aqueous solution of ANS (20 ul). To test the effect of colchicine the cells were first incubated with given concentrations of colchicine for 20 minutes at room temperature (20-25°C) prior to the treatment with con A. Fluorescence intensity and spectrum were measured by an Aminco Bowman spectrofluorometer at 20-25°C immediately after the addition of ANS. The recorded spectra were not corrected for the spectral characteristics of the detection system. With excitation at 360 nm, the emission maximum was 510 nm for ANS in PBS, 470 nm for the probe in 99% ethanol. The observed fluorescence of ANS in the presence of cells was corrected by subtracting the fluorescence signal of the cell suspension measured before adding ANS solution. The binding of ANS to the cells was practically immediate (within 10 seconds) and only a slight increase of fluorescence (less than 5% of the level measured at 10 seconds) was observed by further incubation of the cells with ANS. The binding of ANS was governed to a good approximation by a simple adsorption law between free and bound dyes. Applying the law of mass action under conditions where the amount of bound ANS molecules is much smaller than that of total ANS molecules so as to neglect the former, we obtain,

$$\frac{1}{D} = \frac{\alpha B}{K}\frac{1}{F} - \frac{1}{K}$$

Here D and B are the concentrations of the total (free and bound) dye and total binding sites, respectively. K is the apparent dissociation constant, α is the fluorescence intensity of unit bound dye and F refers to the fluorescence intensity at 470 nm of bound ANS. From the intercept on 1/D axis one can calculate the apparent dissociation constant, when 1/D is plotted against 1/F. Sodium salts of ANS were obtained from Tokyo Kasei LTD. Crystallized con A from nata beans was prepared as we reported previously (10). Colchicine and α-methyl-D-glucoside were purchased from Wako Pure Chemical Industries, LTD.

RESULTS

Effect of con A on the binding of ANS to thymocytes: When ANS was added to the thymocyte suspension, a shift of fluorescence emission maximum from 510 nm for ANS in PBS to 470 nm accompanied by a small increase in fluorescence intensity was observed. This spectral shift and enhancement was very similar to that observed with ANS in 99% ethanol. When thymocytes were incubated with con A at 37°C prior to the addition of ANS, the fluorescence increase of bound ANS at 470 nm was greatly enhanced as shown in Fig. 1. Although con A binds to ANS to give a fluorescence increase at 470 nm, this was actually very small at this concentration of con A and was less than 6% of the total fluorescence intensity of ANS bound to con A-treated thymocytes. This indicates that the con A-induced increase of ANS fluorescence did not result from the fluorescence of ANS bound to con A, but from a change in the binding of ANS to the cells. Fig. 2(A) shows a time course of such an effect of con A-treatment on thymocytes. The effect of con A was observed even after 5 minutes of the treatment. When thymocytes were treated with con A at 0°C, no enhancement of fluorescence of ANS upon the con A treatment was observed. As shown in the same figure, α-methyl-D-glucoside, a specific inhibitor of the binding of con A to the cells, inhibited the effect

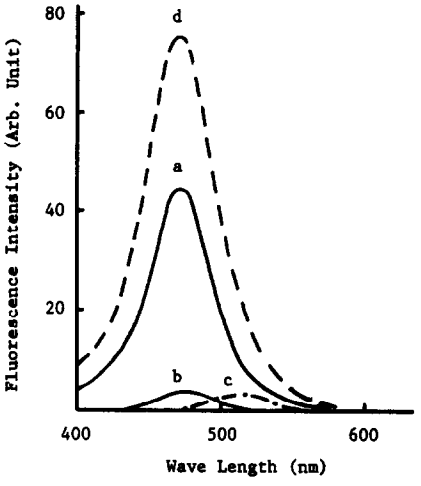

Fig. 1 Fluorescence emission spectra of ANS bound to thymocytes treated with or without con A. Excitation: 360 nm, ANS: 8 x 10^{-6}M. Thymocytes (3 x 10^{6}/ml) were incubated with (a) or without (b) 400 μg/ml con A for 20 minutes at 37°C. Fluorescence of thymocytes alone was subtracted. (c) ANS in PBS, (d) ANS in 99% ethanol.

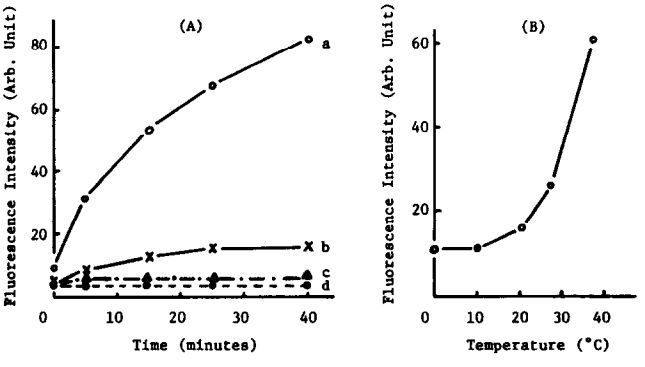

Fig. 2 (A) Time course of the effect of con A-treatment on the fluorescence of ANS bound to thymocytes. Excitation: 360 nm, ANS: 8 x 10^{-6}M. Fluorescence intensity of ANS at 470 nm (emission maximum of the bound ANS) was plotted against the duration of con A-treatment. Thymocytes (4 x 10^{6}/ml) were incubated under the following conditions for indicated periods of time. (a) incubation with 400 μg/ml con A at 37°C, (b) incubation with 400 μg/ml con A in the presence of 0.2 M α-methyl-D-glucoside at 37°C, (c) incubation with 400 μg/ml con A at 0°C, (d) incubation without con A at 37°C. (B) Temperature-dependent profile of the effect of con A-treatment on the fluorescence of ANS bound to thymocytes. Excitation: 360 nm, ANS: 8 x 10^{-6}M. Fluorescence intensities of ANS at 470 nm were plotted against temperatures at which thymocytes (4 x 10^{6}/ml) were incubated with 400 μg/ml con A for 20 minutes.

of con A when the sugar was added together with the lectin for treating the cells. The effect of con A-treatment increased with increasing concentration of the lectin up to about 200 ug/ml, above which concentration the effect reached a plateau. The effect of con A could be observed at a concentration as low as 20 ug/ml. Fig. 2(B) shows temperature-dependence of the effect of con A-treatment. An increase of fluorescence intensity of ANS occurred when the cells were treated with con A at temperatures above 20°C. Since the binding of con A to thymocytes was fairly constant over the temperature range (0-37°C), such temperature-dependent transition of the effect of con A could result from a change of the cells induced after the binding of the lectin.

Apparent dissociation constants for the binding of ANS to thymocytes: The increase in fluorescence of ANS caused by the con A-treatment could be due either to an increase in the number of ANS molecules bound to the cells or, alternatively, to an increase in the quantum yield of bound ANS. Therefore, the statistical average apparent dissociation constant for the binding of ANS to thymocytes was determined under conditions where sufficient amounts of ANS were added so as to neglect the amount of bound ANS compared with the total ANS molecu-

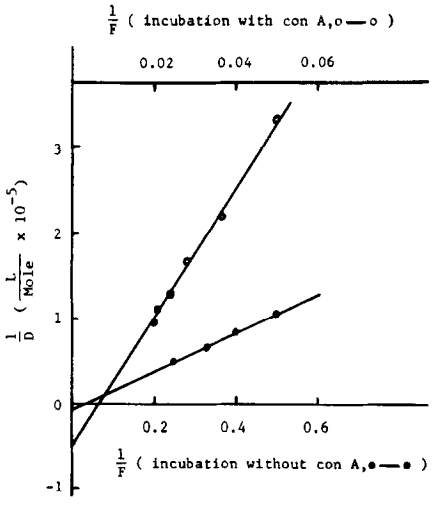

Fig. 3 Determination of the dissociation constants for the binding of ANS to thymocytes. Thymocytes (3 x 10^6/ml) were incubated at 37°C for 20 minutes with or without con A (400 µg/ml) followed by adding various amounts of ANS to the cell suspensions. Reciprocals of the concentrations of added ANS (1/D) were plotted against reciprocals of fluorescence intensities of ANS at 470 nm (1/F). Excitation: 360 nm. Note that the scale for 1/F values when the cells were treated with con A (upper scale of abscissa, o---o) is expanded 10 folds over that for untreated cells (lower scale of abscissa, •---•).

les. The apparent dissociation constant can be obtained from the intercept on 1/D axis when 1/D is plotted against 1/F, where D is the concentration of added ANS and F is the fluorescence intensity of bound ANS (Fig. 3). The apparent dissociation constants thus determined were about 3 x 10^{-5}M and 3 x 10^{-4}M, respectively for con A-treated cells and for untreated cells, indicating a great decrease of the constant by treating the cells with con A. A trial to compare the quantum yield of ANS-fluorescence (fluorescence at infinite cell concentration) for the treated cells with that for untreated cells was unsuccessful, because the fraction of fluorescence signal of cells without ANS increased with increasing concentration of cells, giving less accuracy in the determination of fluorescence of bound ANS at infinite cell concentration.

Effect of colchicine on the increase of ANS-binding by con A-treatment: Colchicine has been reported to inhibit blastformation and thymidine uptake into DNA of con A-stimulated lymphocytes (11, 12). This effect of colchicine is supposed to be due to its action at a very early stage of blastformation and can not solely be ascribed to its action on mitotic spindles. Therefore, we tested the effect of colchicine on the enhancement of ANS-binding to con A-treated cells. Colchicine markedly inhibited the effect of con A-treatment on the fluorescence enhancement of ANS at a concentration as low as 10^{-5}M (Fig. 4). Since colchicine

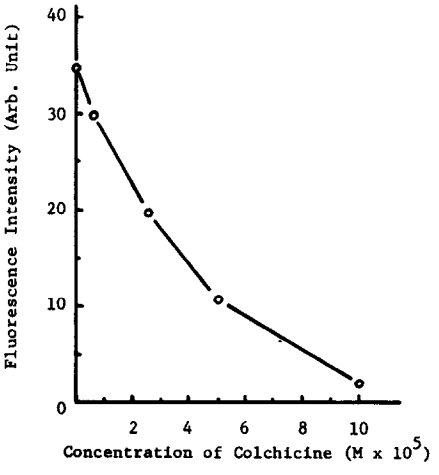

Fig. 4 Effect of colchicine on the stimulation by con A of ANS-binding to thymocytes. Thymocytes (5 x 10^6/ml) were first incubated with indicated concentrations of colchicine at room temperature (20-25°C) for 20 minutes and then treated with con A (400 µg/ml) for 20 minutes at 37°C. Excitation: 360 nm, emission: 470 nm, ANS: 8 x 10^{-6}M.

did not inhibit the binding of con A to the cells as reported (11), the observed effect of colchicine may well be accounted for by its action to eliminate the response of the cells to con A after the binding of lectin molecules.

DISCUSSION

The present results indicate that the fluorescence of ANS bound to thymocytes increases when the cells have been treated with con A. This fluorescence increase is at least due to an increased binding of ANS to the cells (a decrease of dissociation constant) as a consequence of the con A-treatment. Since ANS is partitioned in hydrophobic-hydrophilic interface and is widely used to probe the polarity and viscosity of its microenvironment when bound to proteins or lipids (13), the enhancement of ANS-binding here observed may indicate a change of the chromophore environment of the cells induced by the binding of con A to the cell surfaces. Simple shielding by con A molecules of negative charges on the cell surface that could repel the negatively charged ANS molecules seems not to be a cause of the increased ANS-binding. Because this enhancement of ANS-binding does not occur when the cells have been treated with con A at temperatures below 20°C and in addition it is inhibited by colchicine that does not eliminate the binding of con A to the cells. The localization of bound ANS in thymocytes is not clear at present. The increased fluorescence of ANS upon treatment with con A may result from an enhanced binding of the probe to plasma membranes of the cells or may be due to an increased transport of ANS into the cells. We prefer the former possibility because of the following reasons. When the cells were labeled with ANS and were observed under a fluorescence microscope, ANS fluorescence was seen as periferal rings or a diffuse total label. When thymocytes were homogenized, the fluorescence intensity of ANS increased to a level which was 4 to 6 times that for the intact cells. Furthermore, ANS-fluorescence intensity with the intact cells reached a maximum almost immediately after the addition of the probe to the cell suspension, though it increased thereafter very slightly. These facts lead us to assume that the fluorescence enhancement of ANS induced by the con A-treatment is ascribed to ANS response on the cell surface. Irrespective of the location of bound ANS in the cells, the fluorescence enhancement by the con A-treatment seems due either to an increased binding of ANS to the cell surfaces or to an increased transport of the probe into the cells. Either one of these possibilities could be explained by assuming a change in the properties of plasma membranes of thymocytes.

Such a possible change of properties of plasma membranes of thymocytes seems not to occur solely by the binding of con A to the cell surfaces, since the effect of con A on the fluorescence enhancement shows a sharp temperature-

dependent transition. We suggest that a structural change of plasma membranes could occur at temperatures above 20°C following the binding of con A to cell surfaces. Such a change is induced rapidly after the addition of con A at 37°C, suggesting that the change may occur early in the sequence of events following the lectin binding.

The effect of con A observed here seems closely related to the activity of a colchicine-sensitive system in the cell. Although its identity is not known, this colchicine-sensitive system may be cytoskeletal elements such as microtubules that regulate the distribution and mobility of surface receptors of lymphocytes (11, 12, 14). In this respect, it should be noted that Edelman et al. (12) recently reported that colchicine blocked the mitotic response of lymphocytes to con A at an early stage of blastformation. Barnett et al. (15) and Toyoshima and Osawa (9) described an increase of membrane fluidity induced by the binding of mitogens, suggesting that this is one of the early events in lymphocyte transformation. Our present finding may have something to do with the rearrangement of membrane-receptors and therefore with the change of membrane fluidity. It remains, however, to be determined whether such an early change of con A-treated thymocytes as demonstrated by an increased ANS-binding is responsible for the subsequent activation leading to mitosis of lymphocytes. A preliminary experiment indicates that wheat germ agglutinin, a non-mitogenic lectin, had no appreciable stimulatory effect on ANS-binding to thymocytes.

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