HIGH AND LOW AFFINITY BINDING SITES FOR CONCANAVALIN A ON NORMAL HUMAN FIBROBLASTS *In Vitro*

Martha Feller, Charles Richardson, W. David Behnke, and Eric Gruenstein
Department of Biological Chemistry
University of Cincinnati College of Medicine
Cincinnati, OH 45267

Received April 21,1977

SUMMARY

The binding of high specific activity, radioactive Concanavalin A to cultured normal human fibroblasts was investigated. We report the presence of two classes of Concanavalin A binding sites on the plasma membranes of these cells. These classes of binding sites are distinguished by their affinities for the lectin. Scatchard analysis of the binding data indicates the presence of a class of high affinity sites which are saturated at about 0.25 µg/ml of Concanavalin A. The other, lower affinity binding sites are not saturated until 50-100 µg/ml Concanavalin A levels are achieved. At 4°C the K_a for the high affinity sites varies between 1.5 - 5 x 10^9 M^{-1} depending on the method used to label the Concanavalin A. For the lower affinity sites K_a varies between 1 - 4 x 10^6 M^{-1}. The average number of high affinity sites per cell is 8 x 10^5 representing less than 1% of the total receptor sites for the lectin.

INTRODUCTION

A number of different plant lectins have been shown to bind with a high specificity to glycoproteins or glycolipids of the cell surface (1-5). Very low concentrations of several of these lectins have been shown to agglutinate transformed and protease treated normal cells, whereas agglutination of the normal parental cell generally requires higher concentrations of lectin (6,7). Because transformed fibroblasts are more readily agglutinated by Con A[1] than the corresponding untransformed cells, there have been extensive studies of the differences in the number, distribution, and mobility of Con A receptors on the surfaces of normal and transformed cells grown in culture. The increase in agglutinability shown by transformed vs. normal cells does not appear to reflect a difference in the total number of binding receptors in the cell surface membrane (7,8). Nicolson (9) has obtained data suggesting that this differential agglutinability may be explained by a difference in the mobility of membrane lectin receptors.

Since agglutination and other biological effects induced by lectins such as Con A are expressed at very low occupancy of sites (10), it is apparent that the ability of lectins to initiate these biological effects, by whatever mechanism, results from interaction with only a small percentage of the total number of binding sites for the lectins (11,12). This suggested to us that there might be a small subpopulation of Con A receptors specifically involved in various biological

ISSN 0006-291X

responses to the lectin. In the present study we have examined the binding of the jack bean lectin Con A to normal human fibroblasts with special emphasis on very low lectin concentrations and have found a class of high affinity, low capacity receptors on the surface membrane of these cells. The functional role of these high affinity receptors in agglutination or mitogenesis remains to be determined.

MATERIALS AND METHODS

Chemicals

Na [^{125}I] was obtained from New England Nuclear. [^{63}Ni] was obtained from Amersham Searle. Lactoperoxidase was purchased from Sigma. α-methyl-D-mannopyranoside was purchased from P.L. Biochemicals.

Concanavalin A

Con A was prepared from jack bean meal (Pfaltz & Bauer, Flushing, NY) by the method of Agrawal and Goldstein (13). It was stored as a lyophilized powder at 4°C for up to four months, during which time there was no decrease in activity as determined by polysaccharide precipitation (14).

[^{125}I]Con A with a specific activity of 2 - 3 x 10^4 cpm/μg protein was prepared by the lactoperoxidase method (15). [^{63}Ni]Con A with a specific activity of 2 x 10^4 cpm/μg protein was prepared as described by Koenig, *et al.* (16). Both the [^{125}I]- and [^{63}Ni]-labeled Con A were repurified from the reaction mixture by affinity chromatography on Sephadex G-50 equilibrated with NaCl-P buffer.[2] The column was washed with 100 ml of the same buffer and the bound Con A was selectively eluted with 0.1 M α-methyl-D-mannopyranoside. The eluted material was dialyzed for two days at 4°C against large volumes of NaCl-P buffer. SDS[3] polyacrylamide gel electrophoresis revealed no difference between the labeled and native Con A preparations. [^{3}H-acetyl] Con A with a specific activity of 5.1 Ci/mmole was obtained from New England Nuclear.

Cells

Normal human fibroblasts were obtained from the Institute for Medical Research, Camden, NJ. Cells were grown in monolayer and maintained in a humidified atmosphere at 37°C 5% CO_2-95% air. The culture medium contained Eagle's minimum essential medium (Gibco Cat# F-11) supplemented with penicillin-streptomycin, 100 units/ml, 200 mM Tricine chloride pH 7.4, 24 mM $NaHCO_3$, 1% (V/V)non-essential amino acids and 10% (V/V) fetal calf serum The cells were routinely checked for mycoplasma contamination by standard techniques (17) and were used between the 5th and 20th generations.

Binding of Labeled Concanavalin A to Cells

Cells grown to confluency in Falcon plastic tissue culture dishes (60 mm diameter) were removed from the incubator and placed at 4°C for 5 minutes. Each culture dish was then washed twice with cold NaCl-P buffer and incubated for 1 hour at 4°C in 2 ml of the same buffer containing Con A at the indicated concentrations. After the 1 hour incubation, the cells were washed 5 times with cold NaCl-P buffer and dissolved in 1 ml of 0.1 N NaOH at room temperature. It has previously been determined (data not shown) that 3 washes were sufficient to remove all unbound Con A. Aliquots of the 0.1 N NaOH solubilized cells were counted to determine the amount of Con A which had remained bound to the cells. A second aliquot was removed for protein determinations which were done according to the method of Lowry, *et al.* (18). In the absence of cells no counts were bound to the plate.

[1]Con A (Concanavalin A); [2]NaCl-P buffer (2.6 mM KCl, 1.46 mM KH_2PO_4, 136 mM NaCl and 8 mM $Na_2HPO_4 \cdot 7H_2O$ pH 7.4); [3]SDS (Sodium Dodecyl Sulfate)

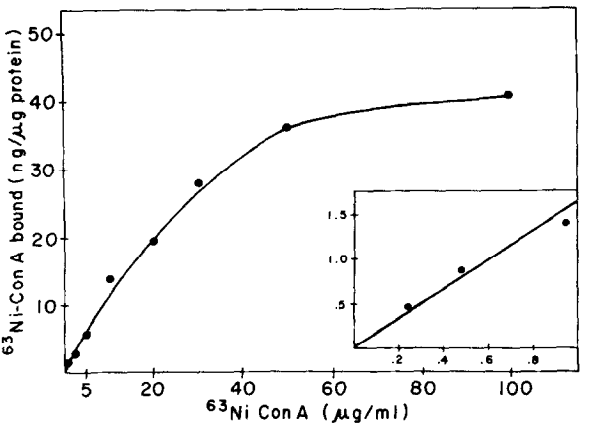


Fig. 1. Binding of [^{63}Ni]Con A to normal human fibroblasts as a function of lectin concentration. Confluent cells were incubated with various concentrations of lectin and the amount of bound Con A was measured as described in Materials and Methods.

Binding of Con A to the cells was analyzed according to the method of Scatchard (19). The amount of Con A bound to the cells (B) was plotted on the abscissa as nmoles of Con A per liter and the ratio of bound to free Con A (B/F) was plotted on the ordinate, where free Con A is expressed as nmoles per liter. High and low affinity association constants were calculated from the negative slopes of the lines obtained, and the number of binding sites were determined by extrapolation of the lines to the intercept with the abscissa (20). There are 2 x 10^3 cells/μg cell protein.

Dissociation of [^{125}I]Concanavalin A-Cell Complex by α-Methyl-D-Mannopyranoside

Normal Human Fibroblasts were incubated with either 50 or 0.1 μg/ml [^{125}I]Con A for 1 hour at 4°C. After this incubation period, the cells were washed 4 times to remove unbound Con A. Dissociation of [^{125}I]Con A from its specific receptors was determined after 10 mM α-methyl-D-mannopyranoside was added for various periods of continuous incubation at 37°C. Dissociated [^{125}I]Con A was removed and acid precipitated using 10% trichloroacetic acid. Measurements of protein concentrations and [^{125}I]Con A remaining bound to the cells were determined as described for the binding experiments.

RESULTS AND DISCUSSION

The binding of [^{63}Ni]Con A to normal human fibroblasts as a function of lectin concentration is shown in Figure 1. The inset shows the binding at concentrations of Con A less than 1 μg/ml. For values above 1 μg/ml, similar results have been obtained by other investigators (7,8). Under the conditions used for this binding study, it has been shown that Con A exists mainly as a dimer of molecular weight of 55,000 (21). If our data are analyzed according to the method of Scatchard (19) as shown in Figure 2, two classes of binding sites are observed. One class of binding sites with an apparent K_a of 1.1 x 10^6 M^{-1} is seen and this is in agreement with the binding

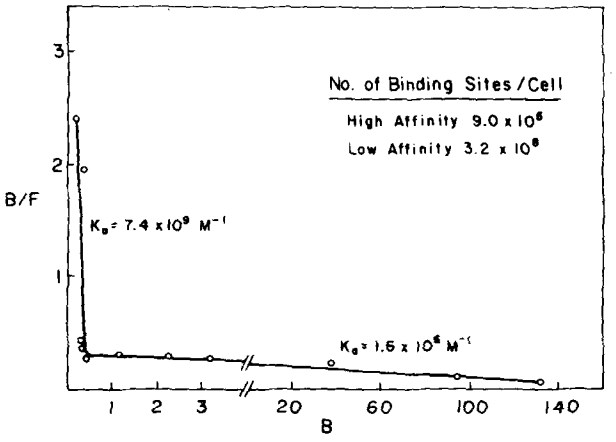


Fig. 2. Scatchard plots of binding of [^{63}Ni]Con A to human fibroblasts. B is expressed as nmoles of [^{125}I]Con A bound per liter. Each data point represents the average of three spearate experiments. Each culture dish contained 314 ± 5 µg of protein. Lines were fit by the method of least squares analysis.

constants previously determined by others (22). However, a second class of binding sites with a significantly higher affinity appears when binding is measured at lectin concentrations less than 1 µg/ml. From the intercept of the line representing the high affinity binding site with the abscissa, we have calculated the number of high affinity sites per cell to be 8 x 10^5. These sites are saturated at a Con A concentration of 0.25 µg/ml. The intercept of the line representing the low affinity sites with the abscissa yeilds a value of 3.0 x 10^8 low affinity sites per cell. The high affinity sites, therefore, represent about 0.3% of the total Con A binding sites. If the total number of Con A binding sites per cell is calculated from the binding curve of Figure 1, a value of 2.8 x 10^8 sites is obtained, in good agreement with the data obtained from the Scatchard analysis.

We were concerned with the possibility of two potential artifacts arising from the use of [^{63}Ni]Con A. First, there was the possibility that small amounts of [^{63}Ni] were dissociating from the Con A and being taken up by the cell. Second, there was the possibility that the chemical properties of Con A were altered by the metal substitution thus generating the high affinity binding. We, therefore, repeated this study using both [^{125}I]-labeled Con A and [^{3}H-acetyl]-labeled Con A. In all cases, Scatchard analysis revealed the presence of both high and low affinity binding sites as previously obtained with [^{63}Ni]Con A. While some differences in the specific binding constants are apparent dependent upon the nature of the chemical

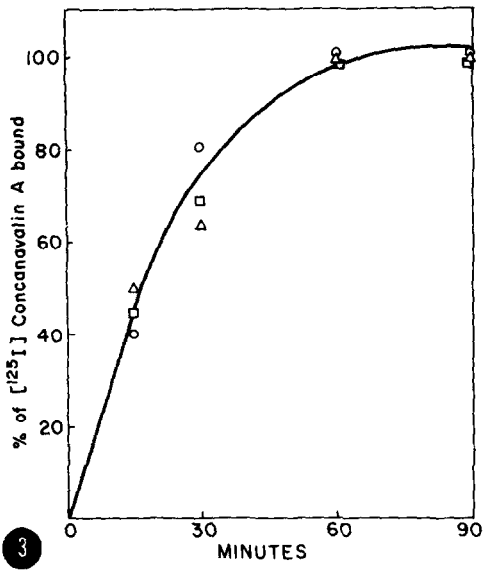
TABLE I. BINDING CONSTANTS FOR CONCANAVALIN A

	High Affinity Receptors (M^{-1})	Low Affinity Receptors (M^{-1})
[^{3}H]Acetyl-Con A	1.0×10^{9}	0.76×10^{6}
[^{125}I]Con A	0.6×10^{9}	3.7×10^{6}
[$^{63}Ni^{2}$]Con A	7.4×10^{9}	1.6×10^{6}

modification of Con A, as shown in Table I, we conclude that the presence of the high affinity binding sites is not due to a special property of the [^{63}Ni]Con A.

We were further concerned that the presence of high affinity low capacity Con A membrane receptors might have been attributable to endocytosis or some other type of non-specific internalization. This possiblity was eliminated on the basis of three criteria. First, all binding studies were done at 4°C, where, over the time period studied, endocytosis should be negligible (23-25). Second, if Con A were being internalized by the cell at these low concentrations, then the amount of Con A associated with the cells should continue to increase with time. The kinetics of Con A binding to the cells were, therefore, studied and the results are shown in Figure 3. This figure shows that normal fibroblasts incubated at 4°C do not continue to accumulate Con A as a function of time when 0.1, 1.0 or 50 μg/ml Con A concentration is used, and in all cases equilibrium is reached within 60 minutes. Third, 90% of Con A bound either to the high or low affinity site was removed after addition of α-methyl-D-mannopyranoside, the sugar which is specific for this lectin. The kinetics of dissociation of Con A bound to the high or low affinity site is shown in Figure 4. This figure indicates that α-methyl-D-mannopyranoside causes a rapid release of bound Con A during the first hour of incubation at 37°C. In addition, all the material removed by α-methyl-D-mannopyranoside was acid precipitable and is therefore not a degradation product of intracellular metabolism of Con A. We conclude that the high affinity binding is not due to the endocytic uptake of bound Con A by the cells.

There are three general models of lectin binding which might account for the presence of these different classes of lectin-membrane affinities: 1) heterogeneous receptors differing in their intrinsic binding affinity (26);



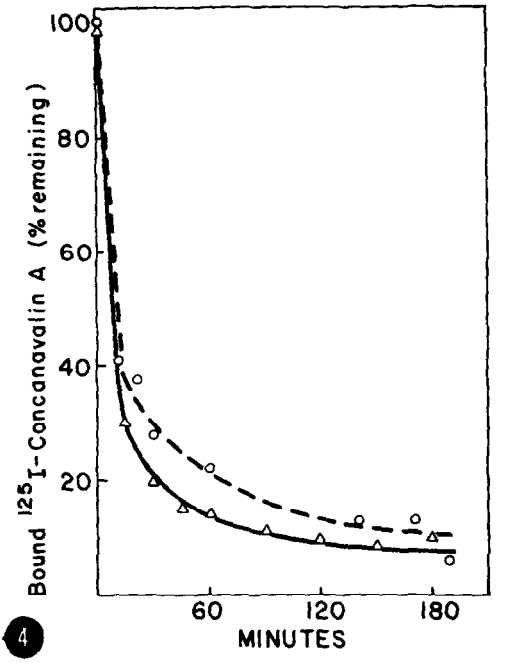


Fig. 3. Binding of ^{125}I Con A to normal human fibroblasts as a function of time. Confluent fibroblasts were washed twice with cold (Ca^{2}--Mg^{2} free) NaCl-P buffer and incubated with 0.1 µg/ml (o——o), 1.0 g/ml (Δ——Δ) or 50.0 µg/ml (□——□) Con A for the indicated time. Bound Con A was measured as described in Materials and Methods.

Fig. 4. Dissociation of ^{125}I Con A from cells by α-methyl-D-mannopyranoside. Normal human fibroblasts were incubated with either 0.1 µg/ml (o——o) or 50 µg/ml (Δ——Δ) Con A for 1 hour at 4°C. Dissociation was measured in the presence of 10 mM α-methyl-D-mannopyranoside after various periods of continuous incubation as described in Materials and Methods.

2) homogeneous receptors displaying site-site interaction of the type called negative cooperativity (27); and 3) homogeneous mobile receptors capable of interacting with a small number of membrane components ("effectors") which, when bound to the receptor, increase its association constant for Con A (28). Our data do not allow us to discriminate among these possibilities. Other investigators have also obtained complex Scatchard plots from the binding of phytohemaglutinin to human lymphocytes (29), from the binding of Con A to thymocytes (30), and from the binding of soybean agglutinin to erythrocytes (31). Only in our studies and in the case of lectin binding to thymocyte microsomal membranes (30) has the question of small amounts of endocytic uptake been carefully eliminated as a possible source of "high affinity binding."

TABLE II. BINDING OF CONCANAVALIN A TO CELLS

CELL TYPE	BINDING SITES PER CELL	SATURATING CON A CONC. µg/ml	K_a, M^{-1}	CONCENTRATION OF CON A FOR MAXIMUM EFFECT		REFERENCE
				MITOGENESIS	AGGLUTINATION	
Normal Human Lymphocytes	1.1×10^7	100	-	10 µg/ml	-	12
Human Lymphocytes (CLL)**	2.8×10^6	100	-	4 µg/ml	-	12
Rat Lymphocytes	3.3×10^6	100	-	2 µg/ml	-	12
Murine Thymus Cells	9.8×10^6	-	1.4×10^6	5%*	-	32
Murine Spleen Cells	9.2×10^6	-	1.3×10^6	5%*	-	32
Murine Bone Marrow Cells	8.9×10^6	-	1.2×10^6	-	-	32
Normal Mouse Thymocytes	$3.0\text{-}9.0 \times 10^7$	50-100	-	3-10%*	-	33
Mouse 3T3	3.0×10^7	100	-	-	1,200-2,000 µg/ml	34
Mouse SV3T3	3.0×10^7	100	-	-	8-16 µg/ml	34
Human Erythrocyte	1.2×10^5	-	3.0×10^6	-	5%*	35

*Values given in these reports as % saturation of specific binding site.

**Chronic Lymphocytic Leukemia (CLL).

Whether the class of high affinity Con A binding sites we have described in normal human fibroblasts is associated with particular biological functions distinct from the functions associated with the low affinity binding sites is not known. It may be of some importance, however, that in those studies which have examined the dose-response relationship for Con A it has usually been found that the concentration of Con A required to elicit responses of agglutination or mitogenesis is such that only a fraction of the total Con A binding sites are occupied (Table II). This stimulation of a biological response at low site occupancy is particularly pronounced in the case of the mitogenic effect of Con A on lymphocytes. We have, therefore, made a preliminary examination of normal human lymphocytes for the presence of a second class of high affinity plasma membrane binding sites and found that these do in fact exist. The association constant is approximately 1.5×10^9 M^{-1} and these high affinity sites are saturated at 0.25 µg/ml which compares favorably with the reported value for onset of mitogenic stimulation by Con A.

ACKNOWLEDGEMENTS

This work was supported in part by research grants to EG from the Muscular Dystrophy Associations of America, the National Institutes of Health No. HL-18553, and the National Foundation-March of Dimes.

REFERENCES

1. Sharon, N., and Lis, H. (1972) Science 177, 949-959.
2. Burger, M. M. (1973) Fed. Proc. 32, 91-101.
3. Lis, H., and Sharon, N. (1973) Ann. Rev. Biochem. 42, 541-574.
4. Janson, V. K., and Burger, M. M. (1973) Biochim. Biophys. Acta 291, 127-135.
5. Chowdhury, T. K., and Weiss, A. K. (1975) Advances in Experimental Medicine and Biology, vol. 55, Plenum Press, New York.
6. Inbar, M., and Sachs, L. (1969) Proc. Natl. Acad. Sci. USA 63, 1418-1421.
7. Ozanne, B., and Sambrook, J. (1971) Nature New Biol. 232, 156-160.
8. Cline, M., and Livingston, D. (1971) Nature New Biol. 232, 155-156.
9. Nicolson, G. L. (1971) Nature New Biol. 233, 244-246.
10. Reisner, Y., Lis, H., and Sharon, N. (1976) Exp. Cell Res. 97, 445-448.
11. Nicolson, G. L. (1973) J. Nat. Can. Inst. 50, 1443-1451.
12. Norogrodsky, A., Biniaminov, M., Ramot, B., and Katchalski, E. (1972) Blood 40, 311-316.
13. Agrawal, B. L., and Goldstein, I. J. (1967) Biochim. Biophys. Acta 147, 262-271.
14. Richardson, C. E., and Behnke, W. D. (1976) J. Mol. Biol. 102, 441-451.
15. Arnd-Jovin, D., and Berg, P. (1971) J. Virol. 8, 716-721.
16. Koening, S. H., Brown, R. D., III, and Brewer, C. F. (1973) Proc. Natl. Acad. Sci. USA 70, 475-479.
17. Barile, M. F. (1973) Tissue Culture Methods and Applications, pp. 729-735, Academic Press, New York.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
19. Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660-672.
20. Chammess, G. C., and McGuire, W. L. (1975) Steroids 26, 538-542.
21. Huet, C., Lonchampt, M., Huet, M., and Bernadai, A. (1974) Biochim. Biophys. Acta 365, 28-39.

22. Kornfeld , S., Adair, W. L., Gottlieb, C., and Kornfeld, R. (1974) Biology and Chemistry of Eucaryotic Cell Surfaces, pp. 291, Academic Press, New York.
23. Steinmn, R. M., and Cohn, A. A. (1972) J. Cell Biol. 55, 186-204.
24. DePeties, S., and Raff, M. C. (1973) Nature New Biol. 241, 257-259.
25. Goldstein, J. L., and Brown, M. S. (1974) J. Biol. Chem. 249, 5153-5162.
26. Scatchard, G., Coleman, J. S., and Shan, A. L. (1967) J. Am. Chem. Soc. 79, 12-20.
27. deMeyts, P., Roth, J., Neville, Jr., D. M., Gavin, III, J. R., and Lesmak, M. A. (1973) Biochem. Biophys. Res. Commun. 55, 154-161.
28. Jacobs, S., and Cuatrecasas, P. (1976) Biochim. Biophys. Acta 433, 485-489.
29. Faguet, G. B. (1976) Leukocyte Membrane Determinants Regulating Immune Reactivity, pp. 65-72, Academic Press, New York.
30. Schmidt-Ullrich, R., Wallach, D. F. H., and Hendricks, J. (1976) Biochim. Biophys. Acta 443, 587-600.
31. Reisner, Y., Lis, H., and Sharon, N. (1976) Exp. Cell Res. 97, 445-448.
32. Stobo, J. D., Rosenthal, A. S., and Paul, W. E. (1972) J. Immunol. 108, 1-17
33. Moller, G., Anderson, J., Pohlit, H., and Sjoberg, O. (1973) Clin. Exp. Immunol. 13, 89-99.
34. Nicolson, G. L. (1975) Advances in Experimental Medicine and Biology, Vol. 55, pp. 153-172, Pelnum Press, New York.
35. Schnebli, H. P., and Bachi, T. (1975) Exp. Cell Res. 91, 175-183.