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INTERACTION BETWEEN *DOLICHOS BIFLORUS* LECTIN AND CHICK EMBRYONIC FIBROBLASTS AT DIFFERENT STAGES OF DEVELOPMENT

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SUMMARY

The interaction between chick embryo fibroblasts and A1-specific blood group *Dolichos biflorus* lectin has been studied at various stages of embryo development. The site number ($(0.26 \pm 0.03) \cdot 10^6$ sites/cell) remains the same during development whereas the affinity constant apparently decreases from 8-day cells onwards. The effects of cell number, temperature and time course on the *Dolichos* binding to fibroblasts were not age dependent. Competitive binding experiments revealed that *Dolichos* receptor sites were distinct from binding sites of *Robina pseudoacacia* lectin and concanavalin A, but partially related to binding sites of *Ricinus* lectin. Thymidine incorporation by fibroblasts in the presence of *Dolichos* lectin was age dependent. It was inhibited in 6-day cells and weakly stimulated in 16-day cells, but not modified in 12-day cells. *Dolichos* lectin effects on embryo fibroblasts were very specific because both binding to cells and effect on thymidine incorporation were blocked by *N*-acetylgalactosamine, the determinant of *Dolichos* lectin, as well as by *Dolichos* antiserum.

INTRODUCTION

The seeds of *Dolichos biflorus* contain a lectin which agglutinates A1-type erythrocytes (1), and specifically precipitates with AI blood group substance [2–3]. Recently, this lectin has been purified and characterized [4, 5]. It is unable to induce either lymphocyte transformation [6] or changes in normal or tumor cell growth [7]. However, *Dolichos* lectin was shown to modify embryonic cell growth as many mitogenic lectins do [8]. In view of these differences in cell susceptibility to *Dolichos* lectin, as determined by cell proliferation, it was interesting to investigate the interaction of *Dolichos* lectin with embryo cells at various stages of development. We report here new information about *Dolichos* lectin and embryo cell interaction. The number of *Dolichos* lectin receptor sites and the binding affinity constant of the lectin were determined using embryo fibroblasts from chick embryos at various stages of development. In addition, the cell response to *Dolichos* lectin was determined by

[^{3}H]thymidine incorporation in fibroblast cultures. The cell response to lectin was shown to be age-dependent and not directly related to the number of *Dolichos* lectin receptor sites.

MATERIAL AND METHODS

Chemicals

[^{3}H]Acetic anhydride (2 Ci/mM) was obtained from C.E.A. (Saclay, France), [^{3}H]thymidine (20 Ci/mM) was obtained from the Radio Chemical Centre, Amersham (England). α-Methylmannopyranoside and *N*-acetylgalactosamine were purchased from Sigma (Saint Louis, Missouri). Other chemical products were the highest purity available from commercial sources.

Lectins

The preparation and characterization of *Dolichos biflorus* [5], *Robinia pseudoacacia* [9] and *Ricinus sanguineus* (tetrameric form: I and dimeric form: II [10]) lectins have been reported previously. Concanavalin A was purchased from Sigma. Lectin homogeneity was tested by polyacrylamide gel electrophoresis.

Labeled Dolichos lectin

Labeled *Dolichos* lectin was prepared according to Miller and Great [11] using [^{3}H]acetic anhydride. The specific activity of the lectin was usually $5 \cdot 10^3$ cpm/μg. Labeled lectin behaved exactly like native lectin in agglutination tests. Lectin solutions were prepared at a concentration of 500 μg/ml in 0.15 M NaCl, 0.01 M $NaHCO_3$ (pH 7.5).

Antisera

Rabbits were injected in the foot pads with 2 mg of *Dolichos* lectin in 0.3 ml of physiologic serum (0.15 M NaCl) emulsified with 0.3 ml of complete Freund's adjuvant. Six weeks later, the rabbits were given four other injections (2 intramuscular and 2 intravenous) of 1 mg/ml of lectin at 24-h intervals. Blood was collected by exsanguination one week after the final injection.

Preparation of cell suspensions

Suspensions of isolated fibroblasts used in the present experiments were obtained according to the technique of Rein and Rubin [12] using mechanical dissociation. Chick embryos were removed from the eggs on the 8th, 10th, 12th and 16th day of incubation. After aseptic dissection of the dorsal muscles in Eagle's medium, tissues were gently ground with a pestle through a cheese cloth (500 μm). The resulting suspension was filtered through a second cheese cloth (60 μm) and washed three times with phosphate-buffered saline (pH 7.4) for binding studies or Eagle's medium for cell culture. The erythrocyte contamination was approximately less than 3 %.

Binding studies

Quantitative binding studies were performed in a centrifuge tube by mixing 0.2 ml of fibroblasts suspension ($1 \cdot 10^7$ cells/ml) with various concentrations of labeled *Dolichos* lectin in 0.1 ml of phosphate-buffered saline (pH 7.4) and 0.1 ml

of bovine serum albumin solution (5 mg/ml). After incubation for 15 min at 4 °C with shaking, the cells were centrifuged and washed three times with phosphate-buffered saline. The pellets were dissolved in 0.2 ml of Soluene (Packard), the solutions were mixed with scintillation fluid and counted in a Packard liquid scintillation spectrophotometer.

Although a rigorous demonstration of reversibility, such as performed by Wurmser and Filitti-Wurmser [13] is lacking, the *Dolichos* binding reaction, as other lectin binding reactions, can be considered to be reversible. Data were plotted according to Steck and Wallach [14]. Affinity constant and site number were calculated taking account of the molecular weight of the lectin and the cell number per assay. To eliminate error due to nonspecific binding of the lectin to the cells the amount of lectin bound to the cell in the presence of 0.1 M *N*-acetylgalactosamine was substracted from the amount of the lectin bound in the absence of the carbohydrate.

Competition studies

To test the relationship between *Dolichos* lectin receptor sites and other lectin receptor sites on embryo fibroblast surfaces, cells were incubated with a saturable concentration of labeled *Dolichos* lectin and the bound radioactivity was determined as the control. In parallel experiments, the same number of fibroblasts was incubated first with a saturable concentration of unlabeled lectin, either *Robinia* lectin, or *R. sanguineus* I, or II lectin or concanavalin A.

Then, the cells were washed and incubated with labeled *Dolichos* lectin at the concentration used in the case of the control. The cells were centrifuged, washed and the bound radioactivity was determined and compared to the bound radioactivity obtained in the case of the control experiment.

Thymidine incorporation into fibroblast cultures

The cultures were primary explant monolayers in 50-mm Petri dishes, in Eagle's medium (3.24 ml) supplemented with 10 % foetal serum. The cultures were grown in humidifical air with 5 % CO_2 at 37 °C. The cultures were seeded at an initial concentration of $5 \cdot 10^5$ cells/ml. The cell cultures were only fibroblasts as determined by phase contrast microscopy. *Dolichos* lectin was added at the beginning of the cultivation at a final concentration varying from 2 to 100 μg/ml. Thymidine incorporation in control cultures and *Dolichos* lectin-treated cultures were assessed by adding [^{3}H]thymidine to each dish at various times of culture (12–72 h).

Two hours later the cells were mechanically harvested from Petri dishes with a rubber policeman and washed twice with cold phosphate-buffered saline. After suspending the cells, an aliquot was counted in a hemocytometer and the viability tested by trypan blue exclusion. The resultant cell suspension was sonicated three times for 15 s and the sonicates were precipitated with 3 ml of cold 10 % trichloroacetic acid. The precipitates were collected on Millipore filters, washed twice with 3 ml of cold 10 % trichloroacetic acid and once with methanol. Radioactivity was monitored in a Packard liquid scintillation spectrophotometer.

Inhibition assays

With sugars. To 0.05 ml of sugar solution in Eagle's minimal essential medium was added an equal volume of lectin solution. After incubation for 30 min at 37 °C,

0.2 ml of cell suspension (at the appropriate cell concentration) was added. Incubations were performed for 15 min (binding studies) or 24 h (thymidine incorporation).

With antiserum. To 0.025 ml of dilution of *Dolichos* antiserum was added 0.025 ml of *Dolichos* lectin solution. After 30 min at 37 °C, the mixture was added to the cell suspensions. Binding of labeled lectin or [^{3}H]thymidine incorporation was determined as above.

RESULTS

(*A*) *Reliability of data*

The range of values from repeated experiments for each point is given on the graphs. The maximum variations, for mean values obtained for a given point in an experiment was 10 % for the binding assay and 5 % for the thymidine incorporation. Maximal variations of the mean values obtained for a given point in the repeated experiments were 13 % for the binding assay and 7 % for thymidine incorporation.

(*B*) *Binding of Dolichos lectin to fibroblasts*

Effect of cell number, temperature and time course. Binding of labeled lectin was linearly related to cell concentration, thus providing evidence that the lectin concentration was not limiting. The quantity of bound lectin increased two-fold from 4 to 37 °C (Fig. 1). Binding of the labeled lectin to cells occurred very rapidly, the kinetic data reaching a maximum with 50 μg of lectin within 15 min at 4 °C (Fig. 2).

Specificity of *Dolichos* lectin binding to embryo fibroblasts was proven since 83 % of the lectins bound was removed by 0.1 M *N*-acetylgalactosamine. These results were not dependent on the age of the embryo fibroblasts.

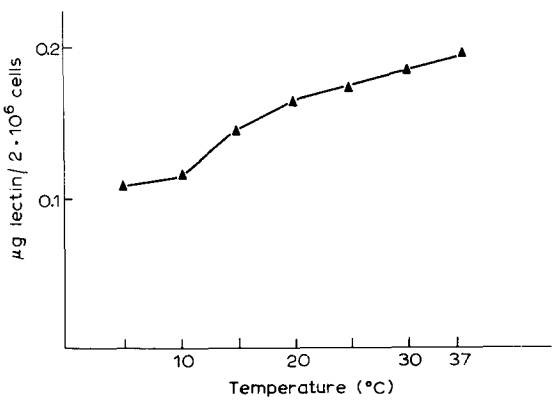

Fig. 1. Effect of temperature on the specific binding of labeled *Dolichos* lectin to 8-day chick embryo fibroblasts. Incubation medium contained $2 \cdot 10^6$ cells and 50 μg of lectin. Ordinate, amount (μg) of ^{3}H-labeled *Dolichos* lectin specifically bound for $2 \cdot 10^6$ cells. Abscissa, temperature (°C).

Quantitative binding of labeled Dolichos lectin to fibroblasts. On the basis of the above results, $2 \cdot 10^6$ cells were routinely incubated at 4 °C with different concentrations of lectin for 15 min in order to calculate the site number and the affinity constant of *Dolichos* lectin for fibroblasts during embryo development.

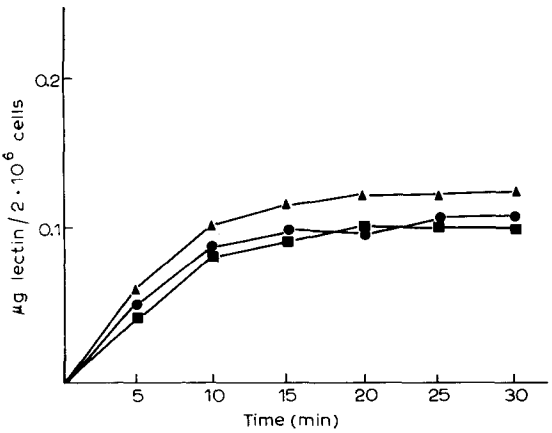

Fig. 2. Time course of specific binding of labeled *Dolichos* lectin to chick embryo fibroblasts at 4 °C. Incubation medium contained $2 \cdot 10^6$ cells and 50 µg of lectin. Ordinate, amount (µg) of ^{3}H-labeled *Dolichos* lectin specifically bound for $2 \cdot 10^6$ cells. Abscissa, time (min); ▲—▲, 8-day cells; ●—●, 12-day cells; ■—■, 16-day cells.

The binding of the lectin to cells was a saturable process with respect to lectin concentration when examined with a lectin amount of less than 30 µg/$2 \cdot 10^6$ cells. The lectin amount required to achieve 50 % saturation was about 10 µg/$2 \cdot 10^6$ cells. The maximum quantity of bound lectin ranged from 0.134 µg/$2 \cdot 10^6$ cells (8-day

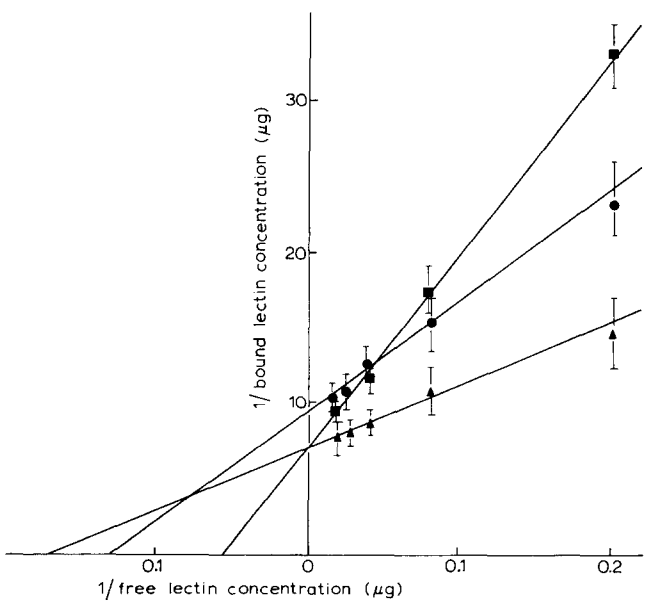

Fig. 3. Binding of labeled *Dolichos* lectin to chick embryo fibroblasts according to Steck and Wallach representation. Cells were incubated at 4 °C for 15 min. Ordinate, 1/bound lectin concentration (in µg); Abscissa, 1/free lectin concentration (in µg); The range of values from repeated experiments for each point is given on the graph. Symbols as Fig. 2.

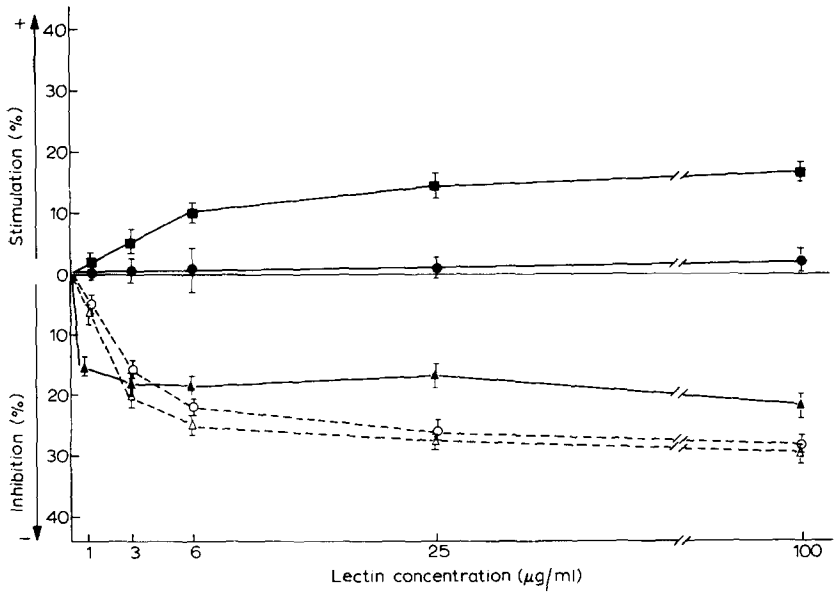

Fig. 4. Dose response curve of [^{3}H]thymidine incorporation by chick embryo fibroblasts exposed to various concentrations of *Dolichos* lectin. Ordinate, % stimulation or inhibition of [^{3}H]thymidine incorporation by cells after 24-h cultures; Abscissa, lectin concentration (μg/ml). 0 % represents the control untreated cells. 6-day cells, △—△; 8-day cells, ▲—▲; 10-day cells, ○—○; 12-day cells, ●—●; 16-day cells, ■—■. The range of values from repeated experiments for each point is given on the graph.

cells) to 0.107 μg/2 · 10^6 cells (12-day cells). The results of *Dolichos* lectin binding to 8-day, 12-day and 16-day chick embryo fibroblasts are presented in Fig. 3.

The number of *Dolichos* lectin receptor sites per cell and the affinity constant were determined by the Steck and Wallach method (Fig. 4). No significant change in site number was noted during cell development ((0.26±0.03) · 10^6 sites/cell) while the affinity constant apparently decreased from 9.5 · 10^6 M^{-1} (8-day cells) to 3.1 · 10^6 M^{-1} (16-day cells).

Lectin competition for Dolichos receptor sites. Dolichos lectin binding to fibroblasts was not changed after the preincubation of fibroblasts with *Robinia* lectin or concanavalin A. On the contrary, the preincubation with *Ricinus* lectin decreased the amount of bound *Dolichos* lectin.

These results were verified in a second set of experiments where the saturation sequence was reversed. No change in the amount of bound *Dolichos* lectin occurred following the incubation of fibroblasts with concanavalin A or *Robinia*, whereas *Ricinus sanguineus* I and II partially removed bound ^{3}H-labeled *Dolichos* lectin (Table I).

(C) Incorporation of [^{3}H]thymidine into fibroblasts in the presence of Dolichos lectin

Thymidine incorporation was dependent on lectin dose and was "age-dependent" (Fig. 4). A lectin dose of 6 μg/ml induced a maximum inhibition of thymidine

TABLE I

COMPETITION OF CONCANAVALIN A AND OF *ROBINIA*, *RICINUS SANGUINEUS* I AND II LECTINS FOR THE *DOLICHOS* LECTIN BINDING SITES ON 8-DAY CHICK EMBRYO FIBROBLASTS

Lectin		% ^{3}H-labeled *Dolichos* bound
First incubation	Second incubation	
^{3}H-labeled *Dolichos*	–	100
^{3}H-labeled *Dolichos*+0.1 M D-GalNAc*	–	12
^{3}H-labeled *Dolichos*	Concanavalin A	93
^{3}H-labeled *Dolichos*	*Robinia*	95
^{3}H-labeled *Dolichos*	*Ricinus sanguineus* I	69
^{3}H-labeled *Dolichos*	*Ricinus sanguineus* II	60
Concanavalin A	^{3}H-labeled *Dolichos*	95
Robinia	^{3}H-labeled *Dolichos*	105
Ricinus sanguineus I	^{3}H-labeled *Dolichos*	53
Ricinus sanguineus II	^{3}H-labeled *Dolichos*	61

* D-GalNAc, *N*-acetyl-D-galactosamine.

incorporation in 6–10 day cells or a maximum stimulation in 16-day cells, after a 24 h culture. Increasing the lectin concentration up to 100 μg/ml did not affect the cell response to *Dolichos* lectin. In addition, thymidine incorporation into 12-day cells was not significantly affected by the lectin at any lectin concentration tested.

The results revealed that thymidine incorporation was time dependent. Fibroblasts were incubated with 6 μg of *Dolichos* lectin/ml, and [^{3}H]thymidine incorporation assessed at various times of the culture. During the first hours of incubation no appreciable difference incorporation of [^{3}H]thymidine occurred in control cells as well as in lectin-treated cells. Following this time, incorporation increased (16-day cells) or decreased (8-day cells) rapidly as compared to control cells and reached a maximum at about 24 h. After this time, the cell response to *Dolichos* lectin slowly decreased. No difference between 12-day control and treated cells was noticed at any time of culture.

Dolichos biflorus lectin had no effect on [^{3}H]thymidine uptake. Advantage was taken of the fact that, as in other systems [15], cooling the cells to 1 °C stops the incorporation of thymidine but does not stop the uptake of the label. Fibroblast cultures were preincubated (37 °C) for 24 h in the presence or absence of lectin and were cooled ato 1 °C. [^{3}H]Thymidine was added for 30 min and its uptake was compared in *Dolichos* treated and control cells. No significant difference was noted between the uptake of the label into treated and control cells.

Inhibition of binding of the labeled lectin and incorporation of [^{3}*H*]*thymidine.* The amount of bound *Dolichos* lectin and thymidine incorporation are markedly decreased in the presence of *N*-acetylgalactosamine. The inhibition of *Dolichos* lectin was dependent on the sugar concentration between 10^{-5} and 10^{-1} molarity. The methylmannopyranoside did not inhibit *Dolichos* lectin.

The binding of *Dolichos* lectin and the action on thymidine incorporation were suppressed by specific *Dolichos* lectin antiserum, but not by non-specific rabbit antisera. The rate of suppression was proportional to the quantity of antibody added

between 1/6 and 1/50 dilutions.

The inhibition of *Dolichos* lectin effects by *N*-acetylgalactosamine and by monospecific anti-serum is age-independent.

DISCUSSION

Agglutinability [16–19] and growth [8, 20] changes of embryo cells mediated by lectins have been shown to be age-dependent. These changes could be related to changes in the number of receptor binding sites during differentiation of the embryo cells [16]. However, during embryo development the number of binding sites had not yet been determined.

Dolichos lectin specifically agglutinates human A1 erythrocytes [1]. This lectin does not change the growth of lymphocytes [6] and of normal and tumor cells [7], but changes the growth of chick embryo fibroblasts according to the developmental stage [20]. As reported here, *Dolichos* lectin binds very rapidly to these cells and a single saturation curve was observed at all embryo ages tested, suggesting homogeneity in the receptor binding sites during differentiation. The number of *Dolichos* lectin receptor sites was significantly less than that observed with concanavalin A and *Robinia* lectin but was similar to *Dolichos* lectin sites number on A1 erythrocytes (Roguet and Bourrillon, unpublished results). The site number was constant during the embryo development which precluded an age-dependent relationship between the lectin sites number and the effect of *Dolichos* lectin on embryo cell growth.

The *Dolichos* lectin receptor sites was found to be distinct from the *Robinia* lectin and concanavalin A receptor sites, but *Ricinus* lectin competed somewhat for *Dolichos* lectin sites suggesting a close steric relationship between these two lectin binding sites on the embryo fibroblast cell surfaces. A similar observation has been reported with mouse S-49 lymphoina cells [10].

The effect of *Dolichos* lectin on embryo fibroblast growth was expressed in parallel, in the [^{3}H]thymidine incorporation, which was inhibited in 8-day cells, weakly stimulated in 16-day cells and not changed in 12-day cells. The time course of *Dolichos* lectin effect on thymidine incorporation in embryo fibroblasts clearly differs from the effect of mitogenic lectins on lymphocytes [21]. Maximum incorporation occurred with lymphocytes after a 72-h culture and with embryo fibroblasts after a 24-h culture. Similar, but non-identical results were reported in the case of DNA synthesis stimulation by concanavalin A in cultures of embryonic neural retina cells [22].

The specificity of the *Dolichos* lectin effect on embryo fibroblasts was proven, since both the binding ability and the effect on thymidine incorporation were specifically inhibited by *N*-acetylgalactosamine or by anti-*Dolichos* lectin anti-serum. As suggested by Oikawa et al. [23] the *N*-acetylgalactosamine residues could play an important role in cell differentiation and organization in embryo development. Inhibition of the *Dolichos* lectin effect by *N*-acetylgalactosamine and anti-*Dolichos* lectin serum is an age-independent process.

Thus the age-dependent effect of *Dolichos* lectin as determined by cell growth and by [^{3}H]thymidine incorporation did not seem to be related to changes either of the number or of the structure of the cell surface sites. Changes in the mobility or distribution of these sites [19] could explain the differential effect of *Dolichos* lectin on embryo cells.

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