BINDING OF CONCANAVALIN A TO NUCLEI OF UNSYNCHRONIZED AND SYNCHRONIZED HELA CELLS

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SUMMARY Purified, intact HeLa cell nuclei were examined for specific Concanavalin A binding using a quantitative binding assay with novel refinements. The result was compared with the specific binding per micron^2 of Con A to RBC's and to HeLa plasma membranes. Con A binding to nuclei isolated from synchronized cells at several phases of the cell cycle was assessed and nuclei sized by Coulter Counter analysis. The Con A binding sites appear to be at the highest density just subsequent to mitosis, decreasing in density between early G_1 and late G_1 and rapidly decreasing between S and G_2. Mechanisms to explain this phenomenon are suggested:

INTRODUCTION Concanavalin A (Con A) has been used to probe the structural properties of membrane systems (1) by study of the distribution and quantity of binding sites on the surfaces of normal and transformed cells. Several techniques have been used to study membranes with this plant lectin: (a) examination of agglutinability to cells (2-4), (b) study of the distribution of surface sugars using ferritin-conjugated agglutinins (5,6), or peroxidase conjugates (7,8) and (c) quantitative binding studies with radioisotopically labelled Con A (3,4,9). Nuclear and plasma membranes from the same cell type have been examined with respect to their comparative Con A binding per unit area. It has been reported (10) that the nuclear and plasma membranes of calf thymocytes bind equivalent amounts of Con A per unit area. Another report (11) concludes that the nuclear and cytoplasmic membranes of rat liver homogenates are not equivalent in Con A binding per unit area; nuclear binding was about 1/30th that of plasma membrane. The contrasting findings may be the result of intrinsic differences between the cell types observed or variations in technical approach.

We have examined Con A binding to HeLa S3 cells, intact HeLa nuclei and

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human type O RH positive erythrocytes (as a control) using an improved binding assay. Combined with studies of nuclear membrane biosynthesis (Michaels, unpublished observations) these studies contribute to the elucidation of the structure and formation of the nuclear surface. The results presented below describe a comparison of specific Con A binding to the plasma membrane and to purified nuclei of HeLa S3 cells. We also compared the specific binding to nuclei purified from synchronized cells at several phases of the cell cycle; nuclei were measured by Coulter Counter analysis.

MATERIALS AND METHODS

Cell culture: HeLa S3 cells were grown at a concentration of 3-6 x 10^5 cells/ml in suspension culture. Joklik's modified Eagles medium with 3.5% each of calf and fetal serum and 10 micrograms/ml Kanamycin was used to maintain the cells at a growth rate of 1 division per 20 hours. Cells were synchronized by thymidine block (12).

Preparation of erythrocytes: Human type O positive erythrocytes were obtained from a single healthy donor. The cells were centrifuged in a Sorvall GLC-2 centrifuge for 5 minutes at 1800 rpm. The serum was removed, the cells were washed three times in phosphate buffered saline, pH 7.3 (PBS, Dulbecco) and refrigerated at 4° C as a 1% solution in PBS. Before each experiment, the cells were washed in PBS to free them from debris and counted in a Bright-Line hemacytometer.

Preparation of HeLa cells: 50 mls of HeLa S3 in suspension culture (ca. 4-5 x 10^5 cells/ml) were centrifuged for 5 minutes at 800 rpm in a Sorvall GLC-2 centrifuge. The medium was decanted and the cells were washed successively by the following regimen: once in Earles's balanced salt solution (EBSS) and twice in PBS to remove glucose. The cells were resuspended in PBS and kept at 4° C until assayed.

Preparation of HeLa S3 nuclei: Following harvesting of 200 ml of cells, they were washed 3 times in EBSS and incubated in RSB buffer (0.01 M NaCl, 1.5 mM $MgCl_2$, 0.01 M Tris-HCL, pH 7.0) for 10 minutes. They were then homogenized in a Dounce homogenizer. A crude nuclear pellet was prepared from 200 ml of cells by pelleting the homogenate at 2000 rpm for 10 minutes in a Spinco SW 27 rotor. The nuclear pellet, resuspended in RSB, was layered on a discontinuous sucrose gradient (0.2 M - 2.0 M sucrose in RSB). The gradients were centrifuged for 60 minutes at 35,000 rpm in a Spinco L5-75 ultracentrifuge with a type SW 41 rotor. The sucrose solution was aspirated from the final nuclear pellets and the tubes were wiped to remove any remaining sucrose-containing buffer. The nuclei were washed 5x and recollected by centrifugation for 5 minutes at 1,500 rpm in the Spinco ultracentrifuge using the SW-27 rotor. Anthrone (13) tests revealed no residual sucrose following 5 washes. The washed nuclei were resuspended in PBS and kept at 4° C until used. Electron microscopy showed that the purified nuclei were intact.

Preparation of Con A: [^{3}H] acetyl Concanavalin A (NEN) was diluted 1:1 by volume with PBS and stored at 4° C in small 0.5 dram glass vials in 50 microliter aliquots. For experiments, 220 microliters of a solution containing 0.5-1.0 mg/ml of unlabelled Con A was added to each aliquot. To remove aggre-

gated material the Con A mixture (labelled and unlabelled Con A) was centrifuged for 1 minute in a Beckman Model 152 microfuge at 9,000 x g. The supernatant of spun Con A (S-Con A) was used for the experiments.

Con A binding assay: Con A binding was assessed by a modification of the method described by Phillips et al. (14). All operations were performed at 0-4°C. Varying volumes (0-230 microliters) of S-Con A were added to incubation tubes and the final volumes standardized by the addition of PBS. At t=0 the material to be assayed was added to the tube containing Con A. Either 1-2 x 10^6 HeLa cells, or 1-2 x 10^5 HeLa nuclei (all suspended in PBS) were added to tubes containing Con A and incubated for 30 minutes. The incubation tubes were shaken intermittently to prevent sedimentation. Following incubation, four 50 microliter aliquots of each sample were layered over 250 microliters of 5% bovine serum albumin cushions in microcentrifuge tubes which had previously been spun for 1 minute to remove bubbles in the albumin. Following a one minute centrifugation of the reacted material, the tips of the microcentrifuge tubes were cut off with a razor blade 0.5 cm from the tip and placed in scintillation counting vials. The pellets were solubilized in 0.5 ml of 10% SDS by agitation and were counted in a Beckman LS-350 scintillation counter with 5 ml of PCS (Amersham) as the cocktail. Each experiment was done 3 times on separate preparations.

Competition assay: Ten fold dilutions of alpha methyl mannopyranoside (α mm) were placed in glass tubes. S-Con A was added to the α mm dilutions and the mixtures were incubated for 35 minutes. At time t=0 the material to be assessed was added as above and incubated for 15 minutes, centrifuged through the albumin and the pellets counted as described above.

Anthrone tests: Anthrone tests were performed on the nuclei following their isolation from sucrose gradients since it has been established that sucrose interferes with the Con A assay. It was determined that 5 washings with PBS were sufficient to remove all detectable sucrose.

Competency test for Con A: All preparations of S-Con A contained 0.55 mg/ml unlabelled Con A in PBS. 0.9 g of Sephadex G-75 was allowed to swell in 150 ml PBS for 24 hours. The Sephadex settled and 75 ml of PBS was decanted. Twenty microliters of the S-Con A to be assayed was added to 180 microliters of PBS. 180 microliters of the prepared Sephadex in PBS was added and the binding reaction allowed to proceed for 15 minutes. Four 50 microliter aliquots were dispensed into microcentrifuge tubes, containing BSA centrifuged for 1 minute, and the pellets were analyzed as above. The competency was determined as the percentage of S-Con A in the reaction tube that bound to the Sephadex, and this test served as a control for various batches of labelled Con A used.

Coulter counting of nuclei: Nuclei which had been purified as above, were washed and diluted to 50,000 nuclei/ml in PBS. Using a channel analyzer the mean volume was determined for nuclei of randomly growing cells and for nuclei isolated from cells at several stages in the cell cycle. We calculated the surface area from the volume assuming that nuclei have a spherical shape.

RESULTS AND DISCUSSION The binding of Con A to the plasma and nuclear membranes has been compared in different systems with disparate results (10,11). Previous studies of Con A binding to the nuclear surface failed to state whether nuclei were intact, or indicate any measurement of the amount of sucrose (which interferes with the binding assay) trapped in the preparations of purified

TABLE I

Molecules of Con A Bound

Material	Molecules of Con A Bound* per cell or nucleus	Per micron^2
RBC (180 microns^2)	$2.75 \pm 0.95 \times 10^6$	1.5×10^4
Unsynchronized HeLa Cells (1100 microns^2)	$8.1 \pm 0.74 \times 10^8$	7.4×10^5
Nuclei from unsynchronized HeLa cells (70 microns^2)	$1.3 \pm 0.3 \times 10^8$	1.9×10^6

* The standard errors of the means are determined from data generated from 3 separate preparations of each material.

nuclei or nuclear envelope. The use of purified nuclear membranes could result in the masking of normally exposed sites or the exposing of internal sites, or both, depending upon whether the vesicles formed right side out or inside out.

We have determined by phase contrast and electron microscopy that the HeLa nuclei purified from homogenates of Dounced cells are intact. Anthrone tests (13) conducted during wash regimens showed that there was significant sucrose contamination (over 50 micrograms/ml) after two washes of purified nuclei. Following our regimen of 5 washes, the sucrose level was below the detectable threshold of the test. A further technical refinement which has not been previously suggested is the centrifugation of the Con A just prior to its use in the assay. We determined that failure to prepare the Con A in this manner allowed aggregates to pellet through the albumin cushion mimicking binding of Con A to the test material and thus yielding spurious results.

Our binding data (Table I) show that human RBC's specifically bind 1-4 x 10^6 molecules per cell or about 1.5×10^4 molecules of Con A per micron^2 which is in agreement with results reported by others for RBC's (14). The binding to

TABLE II

Molecules of Con A Bound*
(Sychronized Cells)

Phase of Cycle	Per Nucleus	Per Micron^2
Late G_1 (104.2 microns^2)	$2.37 \pm 0.15 \times 10^8$	2.2×10^6
S (Synthesis) (124.1 microns^2)	$2.44 \pm 0.1 \times 10^8$	1.97×10^6
G_2 (154.2 microns^2)	$1.22 \pm 0.11 \times 10^8$	7.9×10^5
Early G_1 (97.1 microns^2)	$2.35 \pm 0.25 \times 10^8$	2.42×10^6

* The standard errors of the means are determined from data generated from 3 separate preparations (i.e. 3 separate synchronizations) of HeLa cells.

the HeLa nuclear surfaces is much greater per micron^2 compared to that of the HeLa plasma membrane. This difference may be due to either a greater density of specific binding sites for Con A on the nuclear surface, or to the Con A penetrating the nucleus and binding to either side of the inner membrane, and /or the inner side of the outer nuclear membrane. However, since the nuclei appear to be intact by electron microscopic examination, we believe that the outer nuclear membrane is the site of Con A binding. In all our binding experiments approximately 65-75% of the binding could be competed for by α mm.

When nuclei from different phases of the cell cycle were examined the binding of Con A remained relatively constant (Table II) from late G_1 through S phase at approximately 2.4×10^8 molecules per nucleus and 2×10^6 molecules per micron^2. A significant decline in the number of Con A binding sites (to 1.2×10^8 sites per nucleus and 7.9×10^5 sites per micron^2) occurred between S phase and G_2. Directly following mitosis, at early G_1, the total number and density of Con A binding sites returned to 2.35×10^8 sites per nucleus and 2.42×10^6 sites per micron^2, maximums for the cell cycle.

TABLE III

Volumes of Nuclei from HeLa Cells*

	Volume (Microns^3)	Area (Microns^2)
Unsynchronized HeLa cells	55 (± 3)	70
Late G_1	100 (± 5)	104
S (Synthesis)	130 (± 8)	124
G_2	180 (± 7)	154
Early G_1	90 (± 4)	97

* Volumes measured by Coulter Counter are averages of 4 determinations. The standard errors of the means are from the 4 determinations.

The nuclear volume of unsynchronized and synchronized cells was determined by a Coulter Counter. To our knowledge this is the first report of Coulter Counter measurements of HeLa nuclei or nuclei from any synchronized cells. A dramatic enlargement of the nucleus was observed (Table III) at late S/G_2 phase corroborating the microscopic observations of others. The absolute values however, are considerably smaller than those obtained from microscopic examination. We also noted that when viewed by phase contrast microscopy, the nuclei appeared larger. This is probably due to compression of the nuclei by the cover slip during examination. Other possibilities which may result in discrepancies between determinations from microscopy vs electronic methods have been discussed elsewhere (15).

The data presented support the notion that there are accessible glycoproteins associated with the nuclear surfaces which specifically bind Con A. These Con A binding sites appear to be at the highest density just subsequent to mitosis (early G_1) decreasing in density between the end of S and the onset of G_2. We suggest that there are several possible mechanisms by which this variation in density occurs.

Although it is known from previous work that nuclear membrane biosynthesis occurs throughout S and G_2 (Michaels, unpublished observations) membrane expan-

sion can also occur by a"stretching" mechanism such as seen when nuclei or whole cells are placed in hypotonic medium. Although membrane "stretching" or a lack of Con A receptor biosynthesis may contribute to the decrease in receptor density, it cannot account for the reduction in the number of receptors per nucleus. This reduction must be due to masking or destruction of the receptor sites.

If one assumes that the increase in nuclear surface is accomplished solely by biosynthesis, it is clear that either Con A receptors are not synthesized at a commensurate rate with the increase in nuclear membrane area even during G_1 and S phases, or for some reason these sites do not become available (for binding). Since the total number of binding sites per nucleus drops at G_2, Con A receptors must be masked or destroyed just prior to mitosis. We observed the highest density of Con A binding sites at early G_1. At this stage the total number of Con A binding sites per nucleus is restored, and it appears that these sites become available and/or are synthesized synchronous with the reformation of the nuclear envelope just subsequent to mitosis. Measurements of the specific activities of nuclear envelope associated enzymes during the cell cycle should further clarify the significance of these findings.

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