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ARTICLE *in* BIOCHEMISTRY · FEBRUARY 1975

Impact Factor: 3.02 · DOI: 10.1021/bi00672a029 · Source: PubMed

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Qualitative and Quantitative Interactions of Lectins with Untreated and Neuraminidase-Treated Normal, Wild-Type, and Temperature-Sensitive Polyoma-Transformed Fibroblasts†

Garth L. Nicolson,* Monique Lacorbiere, and Walter Eckhart

ABSTRACT: The lectin receptors of confluently grown hamster BHK, wild type polyoma virus transformed PyBHK, and temperature-sensitive polyoma transformed ts3-PyBHK fibroblasts were investigated using cell agglutination, quantitative [^{125}I]lectin binding, and ferritin-lectin labeling. PyBHK and permissively grown ts3-PyBHK cells agglutinated more strongly with *Ricinus communis* I agglutinin (RCA_I) compared to BHK and nonpermissively grown ts3-PyBHK, although saturation binding of [^{125}I]RCA_I to these cells at 4° resulted in a twofold difference in lectin-binding sites on BHK and nonpermissively grown ts3-PyBHK cells (~1.0–1.3 × 10^7 sites/cell) compared to PyBHK and permissively grown ts3-PyBHK (~0.4–0.6 × 10^7 sites/cell). These cells bound equivalent amounts of [^{125}I]concanavalin A (0.8–1 × 10^7 sites/cell) and [^{125}I]wheat germ agglutinin (1–2.2 × 10^7 sites/cell). Under these binding conditions little endocytosis occurred, as judged by the subsequent release of >90% cell-bound [^{125}I]RCA_I by the RCA_I inhibitor lactose and localization of ferritin-RCA_I exclusively to the extracellular plasma membrane surface. However, if the binding is performed at 22°, only 50% of the bound lectin can be removed by lactose, and ferritin-RCA_I is localized inside the cell within endocytotic vesicles. The relative mobility of RCA_I receptors was examined on ts3-PyBHK cells by the ability of ferritin-RCA_I to induce clustering of its receptors at 22°. RCA_I receptors on permissively grown ts3-PyBHK cells appeared to be more mobile than on nonpermissively grown cells. BHK and PyBHK cells were treated with neuraminidase, and the resulting enzyme-treated cells were assayed for lectin agglutinability and quantitative binding of RCA_I, concanavalin A, and wheat germ agglutinin. Neuraminidase treatment resulted in decreased concanavalin A and wheat germ agglutinability and a slight increase in RCA_I agglutinability. The enzyme-treated BHK and PyBHK cells bound less [^{125}I]wheat germ agglutinin (~2.8 × 10^6 and ~2.2 × 10^6 sites/cell, respectively) and ~2.5 and 6.2 times more [^{125}I]RCA_I (2.5–3 × 10^7 and 3.5–4 × 10^7 sites/per cell, respectively). There was no change in the number of concanavalin A binding sites after neuraminidase treatment. The increase in RCA_I binding sites approximated the decrease in wheat germ agglutinin binding sites indicating that the predominant penultimate oligosaccharide residue to sialic acid on these cells is D-Gal.

The enhanced lectin-mediated agglutinability of cells after neoplastic transformation is well established (reviews: Burger, 1973; Lis and Sharon, 1973; Nicolson, 1974a,b). Although the agglutination properties of transformed cells were originally thought to reflect increased numbers of lectin-binding sites (Burger, 1969; Inbar and Sachs, 1969), several laboratories have found that the total number of lectin receptors does not change after transformation or does not correlate with enhanced cell agglutinability (Arndt-Jovin and Berg, 1971; Cline and Livingston, 1971; Ozanne and Sambrook, 1971; Inbar *et al.*, 1971; Sela *et al.*, 1971; Nicolson, 1973a,1974c; Nicolson and Lacorbiere, 1973; Poste and Reeve, 1974; Phillips *et al.*, 1974). Recently Noonan and Burger (1973a) presented evidence indicating that polyoma-transformed 3T3 fibroblasts have 3.5–5 times the number of concanavalin A (Con A)[1] receptors compared to normal interphase 3T3 cells. Noonan *et al.* (1973) also reported that a temperature-sensitive SV40-transformed 3T3 cell line which expresses a transformed phenotype at 32° (Renger and Basilico, 1972) shows enhanced agglutinability and a four-to fivefold increase in Con A binding sites after shifting the growth temperatures from 39° (nonpermissive) to 32° (permissive). In the present study another temperature-sensitive cell line derived from polyoma-transformed hamster BHK cells (ts3-PyBHK), which also shows growth temperature-dependent lectin agglutinability (Eckhart *et al.*, 1971), was examined for quantitative lectin binding. In contrast to the findings of Noonan *et al.* (1973) with the SV40-transformed 3T3 cell line, the ts3-PyBHK cells bound the same number of Con A and less *Ricinus communis* I (RCA_I) lectin molecules when the cells were grown under permissive conditions, although lectin-mediated agglutinability was enhanced at the permissive growth temperature.

N-Acetylneuraminic acid hydrolase (NANAse) (EC 3.2.1.18) treatment of tumor cells has been reported to increase cell immunogenicity (Currie and Bagshawe, 1968;

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[1] Abbreviations used are: Con A, concanavalin A; NANAse, *N*-acetylneuraminic acid hydro-lyase; PBS, 0.01 M sodium phosphate–0.145 M sodium chloride buffer (pH 7.4); RCA_I, *Ricinus communis* I agglutinin; Tris-NaCl, 0.01 M Tris-HCl–0.01 M calcium chloride–0.145 M sodium chloride buffer (pH 6.5); WGA, wheat germ aggutini.

Bekesi *et al.*, 1971; Simmons *et al.*, 1971) and expose cell surface antigenic sites (Currie and Bagshawe, 1968; Schlesinger and Amos, 1971; Herschman *et al.*, 1972; Rosenberg *et al.*, 1972; Rosenberg and Schwarz, 1974). NANAse treatment of certain tumors and their inoculation into animals have been partially successful in immunizing susceptible hosts against subsequent tumor challenge (Sanford, 1967; Currie and Bagshawe, 1968, 1969). It is obviously important to ascertain what types of cell surface changes occur on tumor cells treated with NANAse, and we report here on the continuation of earlier studies (Nicolson, 1973a) designed to probe the loss of cell lectin receptors and appearance of new receptors caused by the removal of terminal cell surface sialic acid.

Materials and Methods

Cell Cultures and Media. Hamster embryo fibroblasts (BHK, clone 13), a polyoma-transformed derivative (PyBHK; wild type, clone 1A), and a temperature-sensitive polyoma transformed line (ts3-PyBHK, clone 7C) were derived as described by Dulbecco and Eckhart (1970). Cells were grown to subconfluency (contacting) in Nunc petri dishes with Dulbecco's modified Eagles medium containing 10% calf serum (Colorado Serum) at 32 and 39°. The ts3-PyBHK cell line is permissive for the polyoma-transformed state (showing transformed morphology, lectin agglutinability, and growth characteristics) at 32° and nonpermissive at 39°, while the wild type transformed line (PyBHK) expresses the transformed state at both temperatures. Cells were harvested for labeling and electron microscopic studies using 0.02% EDTA in 0.01 M sodium phosphate–0.145 M sodium chloride (pH 7.4) (PBS) or by use of a rubber policeman.

Plant Lectins. Plant agglutinins were purified by affinity chromatography. Con A, obtained as a twice crystallized product (Calbiochem, San Diego, Calif.), was further purified by absorption onto a 2.5 × 30-cm column of Sephadex G-75. After washing with 0.5 M NaCl–0.05 M sodium phosphate buffer (pH 6.5), the agglutinin was eluted with 0.2 M sucrose in the same buffer (Agrawal and Goldstein, 1967) and extensively dialyzed to remove bound saccharides. *R. communis* agglutinins were purified by the procedures of Nicolson and Blaustein (1972) from aqueous extracts of local castor beans. The dialyzed ammonium sulfate precipitated preparation (0–60% ammonium sulfate fraction) of the *R. communis* agglutinins in 0.2 M NaCl–0.05 M sodium phosphate buffer (pH 7.2) was applied to a 4 × 40-cm column of Bio-Gel A 0.5m agarose (Bio Rad). After washing the column with buffer, the agglutinins were eluted in a single peak with the same buffer containing 0.2 M D-galactose. The 120,000 molecular weight lectin (RCA_I) was separated from the 60,000 molecular weight lectin (RCA_{II}) on a 2 × 50-cm column of Sephadex G-100 or Bio-Gel P-100 (Nicolson *et al.*, 1974). Wheat germ agglutinin (WGA) was affinity purified on an ovomucoid-Sepharose column (Burger, 1969, 1970). Crystallized ovomucoid (Sigma Chemical Co.) was coupled to Sepharose by the cyanogen bromide activation method of Cuatrecasas (1970). After extensive washing to remove noncovalently bound protein, the ovomucoid-Sepharose was used to make a 2 × 20-cm affinity column. A crude wheat germ alkaline phosphatase preparation (Worthington) was heat inactivated at 58° for 10 min and then quickly cooled to 4°. After centrifugation to remove precipitated protein, the partially purified WGA preparation was applied to a 2 × 100-cm Sephadex G-75 column and eluted with 1 mM TrisHCl (pH 7.5). The agglutinating fractions were pooled, concentrated, and applied to the ovomucoid-Sepharose column. After washing with buffer, 0.1 M acetic acid (pH 2) was used to elute the agglutinin, and the eluted fractions were immediately neutralized.

Lectins were radioiodinated in the presence of 0.1 M of the appropriate saccharide inhibitor by the iodine monochloride method of McFarlane (1958) and were extensively dialyzed at 4°. Some of the ^{125}I-labeled lectins, notably [^{125}I]Con A, were repurified on affinity columns before use.

Agglutination and Cell Binding of ^{125}I-Labeled Plant Lectins. Cell agglutination assays were performed in plastic trays with 16-mm wells (Linbro FB-54). Cells (2×10^6 per ml) and serial dilutions of agglutinins in PBS were added to each well (total volume, 0.4 ml), and the trays were placed on a rotary table (Henkart and Humphreys, 1970) at 1–2 Hz for 20 min at room temperature. Agglutination was scored by light microscopy (Nicolson, 1973a). Control experiments contained 0.1 M saccharide inhibitors (methyl α-D-mannoside for Con A, lactose for RCA_I, and *N*-acetyl-D-glucosamine for WGA). During these procedures the cells remained >90% viable, as judged by Trypan Blue dye exclusion.

For quantitative labeling, 2×10^6 cells/ml were incubated in plastic tubes (pretreated with 1% bovine serum albumin) for 10 min at 4° with various concentrations of [^{125}I]Con A, -WGA, or -RCA (total volume, 0.3 ml). Occasionally, 2 mM (final) sodium azide was included in the incubation medium, but the results were generally the same with and without azide. After the incubation, the cells were washed twice by centrifugation without azide. After the incubation, the cells were washed twice by centrifugation at 4°, and the final pellet was counted in a Packard γ scintillation counter. For [^{125}I]Con A labeling the final cell pellet was resuspended and transfered to a new tube. Controls were treated in the same manner except that the incubation and washing solutions contained 0.1 M of the appropriate saccharide inhibitor. Specific binding was calculated as the average counts per minute (cpm) of [^{125}I]lectin bound to quadruplicate or triplicate cell samples minus the average cpm of [^{125}I]lectin bound to triplicate controls with 0.1 M saccharide inhibitor present (Nicolson, 1973a; Nicolson and Lacorbiere, 1973). Quantitative calculations of lectin receptors per cell were described by Nicolson (1973a) using 120,000 as the molecular weight of Con A (Kalb and Lustig, 1968) and RCA_I (Nicolson *et al.*, 1974) and 23,500 as the molecular weight of WGA (Nagata and Burger, 1974).

Enzyme Treatment. For NANAse treatment cells were suspended at final concentrations of $0.5–1 \times 10^7$ cells/ml in 0.1 M Tris-HCl–0.01 M calcium chloride–0.15 M sodium chloride buffer (pH 6.5) (Tris-NaCl). Cells previously dialyzed or dissolved in Tris-NaCl buffer for 30–60 min at 37° were treated with 10–250 U/ml (final) *V. cholerae* NANAse (Calbiochem) or 10 U/ml (final) *Cl. perfringens* NANAse (Sigma Type VI, chromatographically pure). Enzyme activities and sialic acid were determined by the Warren (1959) method as described by Herschman *et al.*, (1972). After enzyme treatment cells were washed twice by centrifugation and resuspended in PBS at 4°. NANAse treated cells were judged to be >90% viable by Trypan Blue dye exclusion (Nicolson, 1973a). This treatment results in the release of approximately 60–65% of total cell sialic acid (Nicolson, 1973a) consistent with previous studies (Codington *et al.*, 1970; Rosenberg and Einstein, 1972; Herschman

TABLE I: Agglutination of BHK, PyBHK, and ts3-PyBHK with *Ricinus communis* I Agglutinin.

Cell	Growth Temp (°C)	Cell Agglutination Score at Indicated Conen of RCA_I (μg/ml)[a]						
		10	5	2.5	1.2	0.6	0.3	0.15
PyBHK	39	++++	+++	++	+	+	—	—
ts3-PyBHK	39	+–	+	–	–	–	–	–
BHK	39	+	+	–	–	–	–	–
PyBHK	32	++++	+++	+++	++	+	–	–
ts3-PyBHK	32	++++	+++	+++	++	++	+	+
BHK	32	+	+	–	–	–	–	–

[a] Cell agglutination was scored after 30 min as described under Materials and Methods or in Nicolson (1973a) on a qualitative scale from no agglutination (—) to complete agglutination (++++).

et al., 1973; Rosenberg *et al.*, 1972; Weinstein *et al.*, 1972; Rosenberg and Schwarz, 1974).

Electron Microscopy. Lectins were conjugated to ferritin at 22° by the techniques of Nicolson and Singer (1971, 1974) using the following final concentrations: ferritin, 4–5%; lectins, 1.5–2%; glutaraldehyde, 0.02–0.03%; saccharide inhibitors, 0.1 M. Ferritin-conjugates were affinity purified before use (Nicolson and Singer, 1974). Cells were labeled with ferritin–Con A or ferritin–RCA_I as described (Nicolson and Yanagimachi, 1974) for 7 min at 0°. The cells were washed by centrifugation at 0–4°, and half were fixed in 1.5% buffered glutaraldehyde for 60 min at 4°. The remainder were resuspended in PBS at 20 or 37° for an additional 15 min and were then fixed in glutaraldehyde. The washed, glutaraldehyde-fixed cells were post-fixed in buffered 1% osmium tetroxide at 4°, dehydrated in ethanol–propylene oxide, and embedded in spurr low viscosity resin (Electron Microscopy Sciences). Thin sections were cut with a diamond knife and stained with 2% uranyl acetate prior to examination in an Hitachi HU-12 electron microscope.

Results

Quantitative Lectin Interactions with BHK, PyBHK, and ts3-PyBHK Cells. Polyoma-transformed PyBHK cells agglutinate more strongly with RCA_I compared to BHK cells when cells were grown at 32 or 39°. The ts3-PyBHK cell line agglutinates strongly with RCA_I (similar to PyBHK cells) if the cells are grown at the permissive temperature (32°), but ts3-PyBHK cells agglutinate weakly if grown at 39° (Table I). Previous studies have shown that ts3-PyBHK cells are strongly agglutinated by Con A and WGA if grown at the permissive temperature (Eckhart *et al.*, 1971). Quantitative labeling of BHK, PyBHK, and ts3-PyBHK cells with [^{125}I]RCA_I and -Con A was performed at 4° with cells grown at 32 and 39°. Concentrations of [^{125}I]lectins were used (usually 100–200 μg/ml) that saturate lectin-binding sites within 10 min with low (<15%) background labeling (Figure 1). No differences were found in [^{125}I]Con A saturation binding to BHK, PyBHK, or ts3-PyBHK cells grown at 32 or 39°. The cells bound approximately 8–10 × 10^6 molecules of Con A under saturation conditions. This value agrees favorably with previous studies (Arndt-Jovin and Berg, 1971; Ozanne and Sambrook, 1971; Inbar *et al.*, 1971; Nicolson, 1974c; Phillips *et al.*, 1974). However, BHK cells grown at 32 and 39° and ts3-PyBHK cells grown at 39° consistently and significantly (P <0.001) bound approximately twice as much [^{125}I]RCA_I compared to PyBHK cells and ts3-PyBHK cells

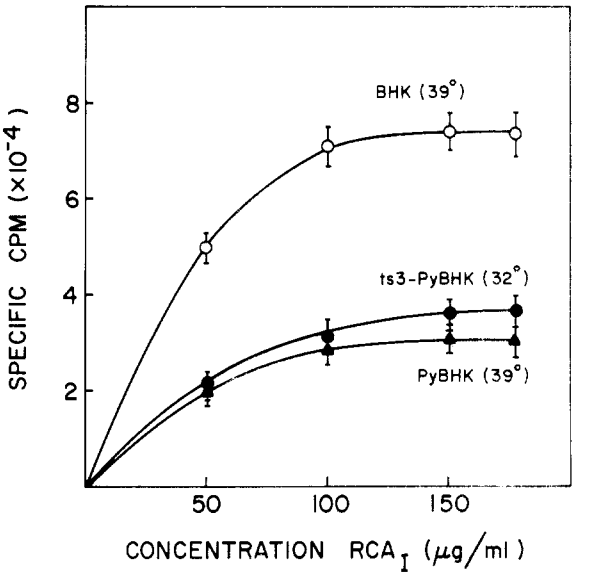

FIGURE 1: Specific binding of [^{125}I]RCA_I to 10^6 hamster fibroblasts during a 10-min incubation at 0–4°. (○) BHK cells grown at 39°; (●) ts3-PyBHK cells grown at 32°; (▲) PyBHK cells grown at 39°. Background labeling in the presence of 0.1 M lactose averaged 6–15% of the raw data.

grown at 39° (Figure 1 and Table II). These cells possess approximately 1.0–1.3 × 10^7 (BHK and ts3-PyBHK, 39°) or 4–6 × 10^6 (PyBHK and ts3-PyBHK, 32°) RCA_I receptors/cell.

Specificity of Quantitative Lectin Binding. As a control for endocytosis of lectin receptors which was reported to modify quantitative lectin binding studies (Noonan and Burger, 1973b), cells were labeled and washed and saccharide inhibitor was added to remove cell-bound lectin. BHK cells labeled at 4° for 10 min with [^{125}I]RCA_I and incubated in lactose solutions for 15 min retained <10% of the lectin (Table III). If BHK cells are labeled with [^{125}I]RCA_I for 30 min at 22°, they bind significantly more lectin (P <0.001), and lactose addition can only remove a portion of the cell-associated lectin (Table III). That at least a portion of this additional cell binding is due to endocytosis of lectin–receptor complexes was demonstrated with ferritin–RCA_I. Cells labeled with ferritin–RCA_I for 10 min at 4° bound the ferritin–lectin conjugate to cell surfaces and lectin-induced endocytosis was not evident (Figure 2a). If, however, cells were labeled at 22° for 30 or 60 min with ferritin–RCA_I, endocytosis occurred and ferritin–lectin could be localized inside some endocytotic vesicles near the cell surface

TABLE II: Quantitative Binding of ^{125}I-Labeled RCA_I to BHK, PyBHK, and ts3-PyBHK Cells Grown at Different Temperatures.

Cell	Expt	Specific cpm of [^{125}I]RCA_I Bound to 10^6 Cells at Saturating Lectin Concentrations[a]: Cells Grown at 32°	Cells Grown at 39°
BHK	1	59,700 ± 3000	60,800 ± 3000
	2	65,600 ± 3500	70,200 ± 3500
	3	61,300 ± 3000	72,100 ± 3500
PyBHK	1	30,400 ± 2000	24,800 ± 2500
	2	24,600 ± 2000	25,200 ± 2000
	3	25,100 ± 2000	29,100 ± 2500
ts3-PyBHK	1	36,100 ± 2500	62,900 ± 3000
	2	23,900 ± 2000	68,900 ± 3000
	3	25,500 ± 2500	73,100 ± 3000

[a] Specific activities of [^{125}I]RCA_I for experiments 1–3 were 6.5–7.2 × 10^{10} cpm/g. Background labeling averaged 6–10% of raw data. Concentration of lectin was 200 μg/ml.

TABLE III: Specificity of Binding of ^{125}I-Labeled RCA_I to BHK Cells.

Lectin Incubation[a]: Time (min)	Temp (°C)	Post-Incubation Additions[b]	Specific cpm of [^{125}I]-RCA_I Bound to 10^6 Cells[c]
10	4		60,100 ± 3000
30	4		69,300 ± 3000
10	4	Lactose	4,200 ± 500
10	22		155,100 ± 5000
30	22		193,600 ± 6000
30	22	Lactose	111,700 ± 5000

[a] Cells were incubated with 100 μg/ml of lectin and washed. For conditions see Materials and Methods. [b] Lectin-labeled cells were suspended in 0.1 M β-lactose for 15 min at 22° and washed twice by centrifutation. [c] Specific activity of [^{125}I]RCA_I was 6.8 × 10^{10} cpm/g.

(Figure 2b). Ferritin–RCA_I labeling was specific as inclusion of lactose in the labeling solution blocked ferritin–RCA_I binding to cell surfaces (Figure 2c). Similar results were obtained with PyBHK and ts3-PyBHK cells. The remainder of the temperature-dependent increase in lectin binding may not be due to endocytosis (Rittenhouse and Fox, 1974) but instead may reflect differences in the mobility of lectin receptors at 4 and 22° and the formation of more multivalently bound lectin molecules at the higher temperature.

Relative Mobility of RCA_I Receptors on ts3-PyBHK Cells. Since ts3-PyBHK cells grown under permissive conditions contain approximately one-half the RCA_I receptors compared to the same cells grown at 39° although they are more agglutinable, we investigated the relative mobility of RCA_I–receptor complexes on these cells with ferritin–RCA_I. Cells grown at 32 and 39° were labeled with ferritin–RCA_I for 7 min at 0°, washed, and then incubated further at 22° for 15 min. After the 0° incubation and after the subsequent 22° incubation, aliquots of cells were fixed in glutaraldehyde and processed for embedding. Thin sections of cells labeled at 0° indicated that little, if any, lectin-induced redistribution occurs in 7 min at this temperature (Figure 3a,b). However, after labeling when the tem-

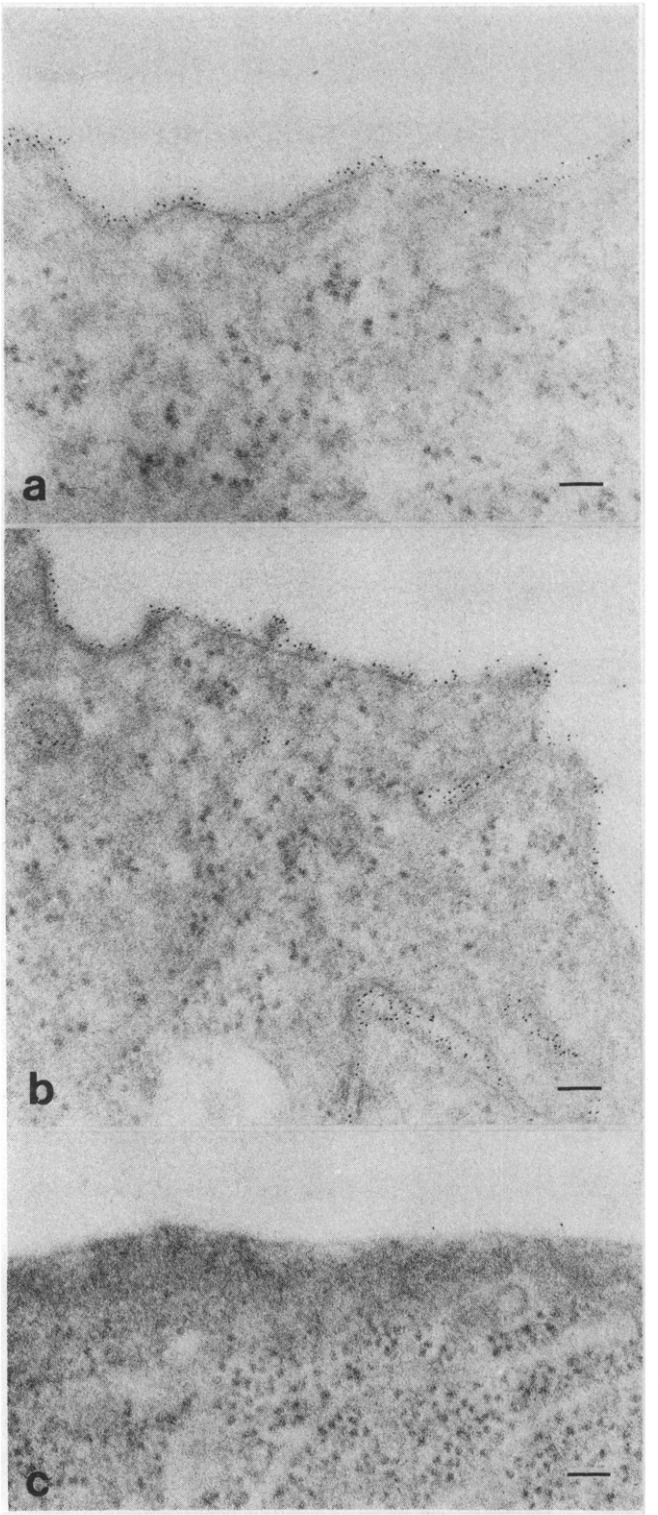

FIGURE 2: (a) BHK cells labeled with ferritin–RCA_I for 10 min at 4°. ×52,500; bar equals 0.1 μm. (b) BHK cells labeled with ferritin–RCA_I for 30 min at 22°. ×52,500; bar equals 0.1 μm. (c) Saccharide control. Legend is the same as in Figure 2b except that 0.1 M lactose was present during the labeling and washing steps. ×52,500; bar equals 0.1μm

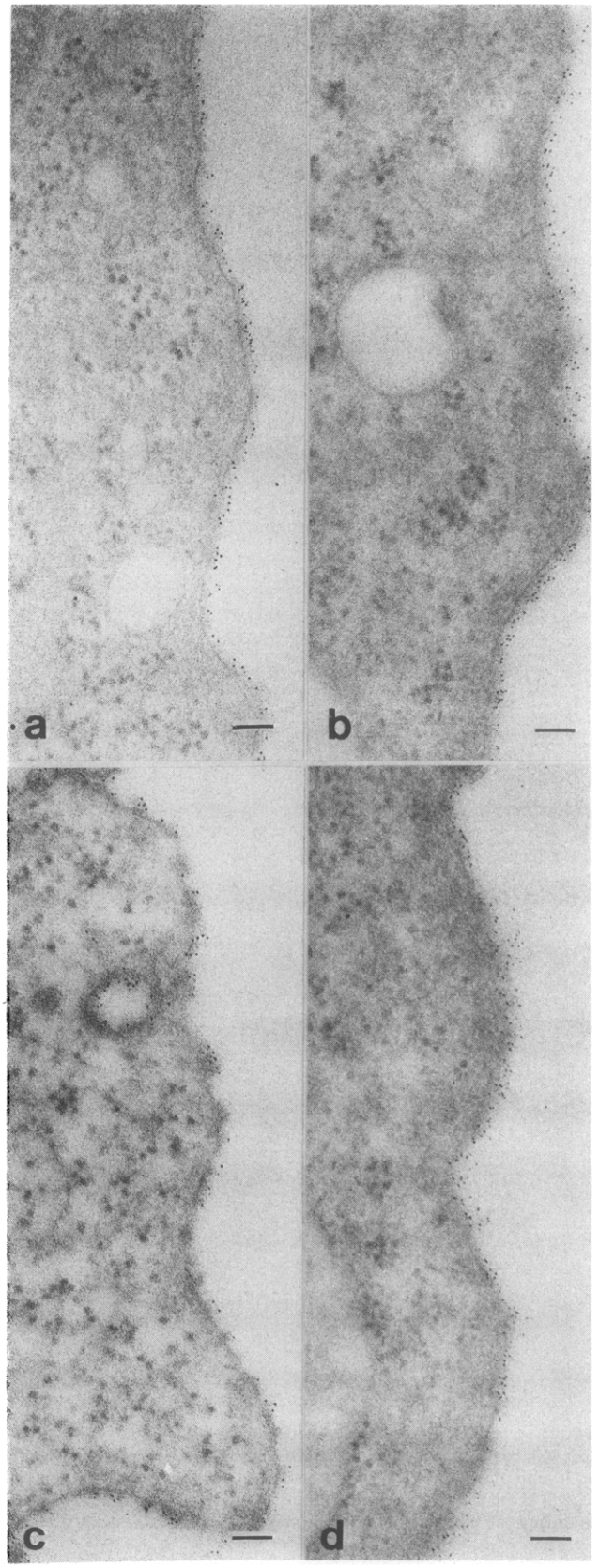

FIGURE 3: (a) ts3-PyBHK cells grown at 32° (permissive) and labeled with ferritin–RCA_I for 7 min at 0°. ×52,500; bar equals 0.1 μm. (b) ts3-PyBHK cells grown at 39° (nonpermissive) and labeled with ferritin–RCA_I for 7 min at 0°. ×52,500; bar equals 0.1 μm. (c) The legend is the same as in Figure 3a except that cells were washed after labeling at 0° and incubated at 22° for 15 min prior to fixation. ×52,500; bar equals 0.1μm. (d) The legend is the same as in Figure 3b except that cells were washed after labeling at 0° and incubated at 22° for 15 min prior to fixation. ×52,500; bar equals 0.1 μm.

perature was shifted from 0 to 22°, lectin-induced clustering of RCA_I receptors occurred. Ferritin–RCA_I was found in a highly aggregated state on ts3-PyBHK cells grown at 32° (Figure 3c), and, although some aggregation of RCA_I receptors occurred on ts3-PyBHK cells grown at 39°, in general it appeared to be more evenly distributed across the cell surface (Figure 3d).

Neuraminidase-Mediated Changes in BHK and PyBHK Lectin Receptors. When BHK and PyBHK cells are treated with NANAse, cell agglutinability increases with RCA_I, but it decreases with WGA and Con A (Table IV). This indicates a possible unmasking of D-Gal-like receptors. Using [^{125}I]RCA_I saturation labeling, this was confirmed. NANAse treated BHK and PyBHK cells have 2.5–3 × 10^7 and 3.5–4 × 10^7 RCA_I binding sites/cell, respectively (Table V). These represent ~2.5 and ~6.2 times increases in RCA_I receptors on NANAse treated BHK and PyBHK cells. In contrast, the number of WGA binding sites on BHK and PyBHK cells (~2.2 × 10^7 sites/cell) decreased after NANAse treatment to ~2.8 × 10^7 and ~2.2 × 10^6 binding sites/cell, respectively. Con A receptors remained numerically unchanged after NANAse treatment (Table V). The decrease in WGA receptors on NANAse treated BHK and PyBHK cells very closely approximated the increase in RCA_I receptors. Since WGA binds *N*-acetyl-D-glucosamine and sialic acid (LeVine *et al.*, 1972), the loss of WGA receptors and the concomitant increase in RCA_I receptors specific for terminal D-Gal residues (Nicolson *et al.*, 1974) indicate that an overwhelming proportion of the penultimate oligosaccharide residues to terminal sialic acid unmasked by NANAse in our experiments are probably D-Gal residues.

Discussion

Conflicting results have been obtained by investigations studying the number of lectin-binding sites on normal and transformed cells. It was originally proposed that transformation results in increased numbers of lectin-binding sites in subconfluent cell cultures (Burger, 1969; Noonan and Burger, 1973a; Noonan *et al.*, 1973). We and others (Arndt-Jovin and Berg, 1971; Cline and Livingston, 1971; Ozanne and Sambrook, 1971; Inbar *et al.*, 1971; Nicolson, 1973a,1974c; Phillips *et al.*, 1974) found different results when lectin receptors on confluently grown normal and transformed fibroblasts were compared. Indeed, using [^{125}I]RCA_I we found that PyBHK cells and ts3-PyBHK cells (grown under permissive conditions) possess approximately one-half the number of RCA_I receptors compared to untransformed BHK cells or ts3-PyBHK cells (grown under nonpermissive conditions). No differences were found in the number of Con A or WGA receptors on these cells. Noonan and Burger (1973a) found that at 0° 3T3 fibroblasts bind a maximum of 6.6 × 10^5 and Py3T3 and SV3T3 cells bind approximately 1.3 × 10^6 [^{3}H]Con A molecules, respectively. This can be compared to the results of Arndt-Jovin and Berg (1971) in which 3T3 and SV3T3 cells labeled at 0° bound 1–1.3 × 10^7 molecules of [^{125}I]Con A, and those of Inbar *et al.* (1971) who found that normal and SV40-transformed hamster cells bound approximately 3 × 10^7 molecules of [^{63}Ni]Con A at 4°. Similarly, the data here showed that BHK, PyBHK, and ts3-PyBHK cells bound approximately 0.8–1 × 10^7 molecules of [^{125}I]Con A at 4°. We also found in accord with the data of Noonan and Burger (1973b) that quantitative lectin labeling at 22° results in significant endocytosis of cell-bound lectin molecules. This

TABLE IV: Agglutination of Untreated and Neuramindase-Treated BHK and PyBHK cells with Con A, Wheat Germ, and *Ricinus communis* I Lectins.

Lectin	Cell ± NANAse[a]	Cell Agglutination Score at Indicated Concn (μg/ml)[b]							
		2700	1350	675	340	170	90	45	20
WGA	BHK	++	+	+	−	−	−	−	−
	BHK–NANAse	±	−	−	−	−	−	−	−
	PyBHK	++++	++++	+++	+++	++	++	+	±
	PyBHK–NANAse	+	+	±	−	−	−	−	−
		1600	800	400	200	100	50	25	12
Con A	BHK	+	+	±	−	−	−	−	−
	BHK–NANAse	±	−	−	−	−	−	−	−
	PyBHK	++++	++++	+++	+++	++	++	+	±
	PyBHK–NANAse	+++	++	+	±	−	−	−	−
		15	7.5	3.7	1.8	0.9	0.45	0.2	0.1
RCA_I	BHK	++	+	+	−	−	−	−	−
	BHK–NANAse	++	++	+	±	−	−	−	−
	PyBHK	++++	++++	+++	+++	++	+	+	−
	PyBHK–NANAse	++++	++++	++++	++++	+++	++	+	±

[a] Cells were treated with neuraminidase for 30 min at 37° described under Materials and Methods. [b] Agglutination was scored as described in Table I.

TABLE V: Quantitative Binding of ^{125}I-Labeled Concanavalin A, Wheat Germ, and *Ricinus communis* I Lectins to Untreated and Neuraminidase-Treated BHK and PyBHK Cells.

^{125}I-Labeled Lectin[a]	Cell ± NANAse[b]	Specific cpm of [^{125}I]-Lectin Bound to 10^6 Cells at Saturating Lectin Concn[c]
WGA	BHK	54,500 ± 2,510
	BHK–NANAse	7,800 ± 500
	PyBHK	53,900 ± 2,500
	PyBHK–NANAse	4,900 ± 600
Con A	BHK	116,200 ± 9,000
	BHK–NANAse	105,300 ± 10,000
	PyBHK	104,600 ± 9,000
	PyBHK–NANAse	108,100 ± 9,000
RCA_I	BHK	65,500 ± 2,500
	BHK–NANAse	159,300 ± 5,000
	PyBHK	36,900 ± 2,000
	PyBHK–NANAse	232,900 ± 8,000

[a] Concentrations of lectins were 150 μg/ml (WGA, RCA_I) or 200 μg/ml (Con A). [b] Cells were treated with neuraminidase for 30 min at 37° as described under Materials and Methods. [c] Specific activities were as follows: [^{125}I]WGA, 5.01×10^{10} cpm/g; [^{125}I]Con A, 2.0×10^{11} cpm/g; [^{125}I]RCA_I, 7.06×10^{10} cpm/g. Background labeling averaged 7–13% of raw data.

was demonstrated by the incomplete removal of cell-bound RCA_I by lactose after labeling at 22° and by the endocytosis of ferritin–RCA_I under similar conditions. However, the labeling conditions chosen here for quantitative estimation of lectin binding sites (100–200 μg/ml of lectin for 10 min at 4°) do not permit significant levels of endocytosis. Cell-bound lectin was >90% removed by its specific inhibitors, and ferritin–lectin conjugates remained at the cell surface during the incubation period. A subsequent shift of incubation temperature of lectin-labeled cells from 4 to 22° or 37° resulted in lectin-induced endocytosis (data here and Nicolson, 1974c; Oliver *et al.*, 1974). The absence of significant endocytosis argues against the proposal (Noonan and Burger, 1973a,b) that the lack of differences between the number of lectin receptors on normal and transformed cells in previous quantitative lectin labeling experiments is due to endocytosis. Several investigators have used labeling conditions that are probably sufficient to prevent massive lectin-induced endocytosis (*e.g.*, Arndt-Jovin and Berg, 1971). One possible explanation for the results of Noonan and Burger (1973a) and Noonan *et al.* (1973) is that under their labeling conditions, they selectively saturated a class of Con A receptors on transformed cells that have characteristically high binding constants. That such high avidity sites may exist on cells is supported by Cuatrecasas' (1973) findings that fat cells have a heterogeneity of Con A binding site affinities. If transformed cells have a special class of high avidity Con A receptors accounting for, say, <10% of the total Con A sites, the high avidity sites could account for the data of Noonan and Burger (1973a) and Noonan *et al.* (1973) and would be consistent with the data here and elsewhere.

It is apparent from several studies that the number of lectin receptors on a cell bears no simple relationship to its agglutinability by lectins (Arndt-Jovin and Berg, 1971; Cline and Livingston, 1971; Ozanne and Sambrook, 1971; Sela *et al.*, 1971; Inbar *et al.*, 1971, 1973; Nicolson, 1973a,1974a,c; Poste and Reeve, 1974). Cell agglutination is a complex phenomenon that is determined by a variety of complicated interrelated factors (Nicolson, 1974a,b). These include: (1) biochemical nature of the agglutinating molecule including the saccharide-binding constant, valency, net charge, number of receptors, etc.; (2) the number of *available* cell-bound agglutinin molecules that are sterically capable of interacting with another cell's surface receptors; (3) the mobility of the receptors and their ability to undergo

clustering to form high receptor density areas; (4) the charge repulsive forces of the cells; and (5) cell surface structures such as microvilli that can present specialized surface regions to adjacent cells, etc. (see Nicolson, 1974a–c). We examined the mobility or RCA_I receptors on ts3-PyBHK cells grown at 32 and 39° and found that, in general, the RCA_I receptors on ts3-PyBHK cells grown at 32° were relatively more mobile as judged in lectin-induced clustering experiments, although this result was not as clear as in other studies (Nicolson, 1971, 1973a,b; Bretton *et al.*, 1972; Martinez-Palomo *et al.*, 1972; Rosenblith *et al.*, 1973; Rowlatt *et al.*, 1973; Garrido *et al.*, 1974). Since these cells are more agglutinable and exhibit fewer RCA_I receptors than when they are grown at 39°, it is possible that enhanced mobility of RCA_I receptors may play an important role in the differential agglutinability of these cells. Enhanced mobility of lectin receptors after transformation has been seen in other normal and tumor cell systems (Inbar and Sachs, 1973; Rosenblith *et al.*, 1973; Nicolson, 1973b,1974a; Huet and Bernhard, 1974; Garrido *et al.*, 1974), but it cannot explain differential agglutination in every normal-transformed cell system (de Petris *et al.*, 1973; Roth *et al.*, 1973; Huet and Bernhard, 1974).

The quantitative binding studies here with $[^{125}I]RCA_I$ agree nicely with data indicating that BHK and ts3-PyBHK (grown at 39°) cells have an exposed galactoprotein that is inaccessible or missing in PyBHK or ts3-PyBHK (grown at 32°) cells (Gahmberg *et al.*, 1974). Using a galactose oxidase–$[^3H]$borohydride labeling technique, these authors were able to label cell surface glycoproteins containing terminal D-Gal and separate the labeled glycoproteins on sodium dodecyl sulfate polyacrylamide electrophoresis gels. A glycoprotein of approximately 200,000 molecular weight (galactoprotein *a*) was one of the major D-Gal-containing surface components, and its expression was dependent on the growth and transformation state of the cells. Galactoprotein *a* was expressed on BHK or ts3-PyBHK (grown at 39°) cells only at cell confluency, and its reduction in content or accessibility by protease treatment led to the proposal that exposed galactoprotein *a* might function as an important receptor in growth control (Gahmberg *et al.*, 1974). Since Gahmberg *et al.* (1974) found that galactose oxidase–$[^3H]$borohydride did not label lactosyl ceramide of either normal or transformed cells, there is a good chance that cell surface glycolipids may not protrude far enough from the cell surface for lectin interaction. If this assumption is correct, then the difference in RCA_I binding sites between ts3-PyBHK cells grown at 32 and 39° ($\sim 6 \times 10^6$ sites) could represent the upper limit to the addition or exposure of galactoprotein *a* plus other minor galactoproteins on ts3-PyBHK cells when shifted from permissive to nonpermissive growth conditions.

Finally, inoculation of animals with NANAse treated tumor cells is known to offer some protection against subsequent tumor challenge (Currie and Bagshawe, 1968; Simmons and Rios, 1971; Bekesi *et al.*, 1971). This effect is known to be related to the immunological rejection of NANAse treated tumor cells (Simmons and Rios, 1973) due to their enhanced immunogenicity (Bagshawe and Currie, 1968; Sanford and Codington, 1971; Simmons *et al.*, 1971; Hughes *et al.*, 1973). Cells treated with NANAse possess "unmasked" antigens which probably account for their changed immunological properties (Sanford and Codington, 1971; Rosenberg and Rogentine, 1972; Simmons and Rios, 1973; Hughes *et al.*, 1973; Rosenberg and Schwarz, 1974). It was found here and elsewhere (Pardoe *et al.*, 1970; Nicolson, 1973a; Hughes *et al.*, 1973) that NANAse treated cells are more agglutinable with RCA_I. Quantitative lectin binding data indicate that the loss of WGA binding sites from NANAse treated cells almost equalled an increase in RCA_I receptors, while there was no change in Con A receptors. Since WGA can interact with sialic acid (LeVine *et al.*, 1972) while RCA_I reacts with terminal galactose residues, we tentatively conclude that the overwhelming proportion of the penultimate residues unmasked by NANAse are D-Gal. Since RCA_I favors β-D-Gal residues (Nicolson *et al.*, 1974), these penultimate residues are probably β linked to the next sugar residue. In addition, more of these new receptors are uncovered on NANAse treated transformed cells compared to normal cells. Studies are currently in progress to determine the most probable saccharide sequence of common tumor cell surface sialo components and to isolate membrane components containing NANAse unmasked D-Gal by affinity chromatography on RCA_I containing affinity columns. It is hoped that these studies will provide further chemical and immunological information on tumor rejection.

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