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Cell Surface Changes Accompanying Viral Transformation: *N*-Acetylneuraminic Acid Ectotransferase System Activity[1] (38833)

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The presence of glycosyl transferases on the plasma membrane of chick embryo neural retinal cells (1), L1210 leukemia cells (2), chick embryo fibroblasts (3), 3T3 mouse fibroblasts (4), and human blood platelets (5), have been demonstrated. It has been suggested that surface glycosyl transferases are at least partially responsible for cell-to-cell (6) and platelet-to-collagen adhesion (5). Other possibilities include facilitation of cell recognition by ectotransferase systems or expression of residual Golgi glycosyl transferase activity present during plasma membrane biogenesis. Conflicting reports on the levels of glycosyl transferase in transformed vs normal tissue culture cells have appeared. Both increased (3) and decreased (7) levels of enzyme activity have been correlated with transformation. This inconsistency suggests that either transferase activity is not correlated with transformation or that some variable influencing enzyme activity has not yet been determined and controlled. The fact that extensive changes in the glycoprotein composition of the cell surface occur in transformation is well documented (8). In an attempt to clarify the relationship between transformation and the ectotransferase system, in the present study the surface sialyl ectotransferase systems of normal and transformed chick embryo fibroblasts have been compared at different reaction pH values and temperatures and for ion sensitivity.

Material and Methods. Cell culture and viruses. Primary cultures of chick embryo cells were prepared and maintained in F-12 medium as previously described (9). Cultures were refed with fresh medium every 48 hr. The chick cells, derived from a leukosis-free flock tested for uniform susceptibility to RSV-subgroup A and B viruses, were infected with the Schmidt-Ruppin strain of subgroup A (SR-RSV) supplied by Dr. H. Hanafusa. The cultures were split on the second day after infection and used for biochemical studies on the fourth day, when over 90% of the cells appeared visibly transformed. The cells were harvested by gentle scraping with a rubber policeman. Only confluent cultures were used so that effects of cell contact would be the same in all cultures.

Enzyme treatment of cells. After harvesting, the cells were washed once with Dulbecco's medium minus serum. The intact, unaltered fibroblasts or cells treated with neuraminidase were used as acceptors in the sialyl transferase assay. For neuraminidase treatment, 50.0 mg (wet wt) of cells were suspended in 1.0 ml of Dulbecco's medium minus serum (pH 7.0) and incubated at 37°C with 0.04 units of *Clostridium perfringens* neuraminidase (EC 3.2.1.18) purchased from Worthington Biochemical Corp. (Freehold, NJ). After 20 min the cells were removed from the incubation medium by room-temperature centrifugation at 700*g* for 5 min. The cells were washed twice with fresh medium.

Cell viability. After incubation of the cells with varying concentrations of neuraminidase, cell viability was determined by the Trypan blue exclusion test (0.1% Trypan blue dissolved in physiological saline).

Transferase assay. The cell pellet with or without prior neuraminidase treatment was resuspended in 450 μl of Dulbecco's medium minus serum. Various ions were added to the cell suspension in a volume of 50 μl. For controls minus ions, an additional 50 μl of medium was added. To the 0.5 ml suspension, 100 μl of a 3.3 μCi/ml solution of CMP-^{14}C-NANA (sp act 100 Ci/mole; New

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TABLE I. INFLUENCE OF CATIONS ON THE SIALIC ACID ECTOTRANSFERASE SYSTEM ACTIVITY OF INTACT CELLS.[a]

Cation	Concentration	Cell line	
		CEF	SR-RSV-CEF
Mn^{2+}	8 m*M*	115 ± 10	150 ± 20
Mg^{2+}	8 m*M*	103 ± 2	108 ± 1
Ca^{2+}	8 m*M*	91 ± 6	96.9 ± 6

[a] Intact, untreated cells in Dulbecco's medium (pH 7.0) were used in the experiments, as described in Experimental Procedures. Results are expressed as percentage of activity present without cation addition, and are the average ± 1 SD of five experimental determinations on separate cell cultures.

England Nuclear) was added. This concentration of substrate was sufficient to produce maximum enzyme activity. The cell suspension, total volume 0.6 ml, was incubated for 15 min at 37°C unless otherwise specified. To terminate the incubation, excess cold Dulbecco's medium was added, and the cells were removed by centrifugation at 700*g* for 5 min. Cell protein was precipitated with 3.0 ml of 1% phosphotungstic acid in 0.5 *N* HCl and centrifuged out of solution. After washing with 3.0 ml of 10% trichloroacetic acid followed by ethanol:ether (2:1, v/v), the precipitate was dissolved in 1 *N* NaOH. Radioactivity was determined by liquid scintillation counting. Results were expressed as cpm per mg of Lowry protein.

Protein determination. Protein was determined by the method of Lowry *et al.* (10) utilizing bovine serum albumin as a standard.

Results. Cation effect on the activity of the sialyl ectotransferase system. The incubation of intact, viable SR-RSV-CEF cells with 8 m*M* Mn^{2+} resulted in a marked stimulation of sialyl ectotransferase system activity. Mg^{2+} had a slight stimulatory effect, and Ca^{2+} had a small inhibitory effect. As seen from Table I, qualitatively the same effect on the activity of the ectotransferase system with ion addition was seen with normal CEF cells. However, addition of Mn^{2+} only slightly increased the incorporation of ^{14}C-NANA and addition of Ca^{2+} to the incubation medium produced more inhibition in the CEF cells than in the SR-RSV-CEF cells.

Cation effect on the sialyl ectotransferase system of neuraminidase-treated cells. When the cells were treated with neuraminidase before enzyme assay, ectotransferase system activity was increased two to three times. There was no statistical difference between the percentage increase in activity after neuraminidase of normal and transformed cells. Without ion addition, the cpm per mg of cell protein were greater in the normal CEF cells (Table II). After addition of Mn^{2+} to the assay system, this difference was insignificant. The ion sensitivities of the ectotransferase of neuraminidase-treated CEF and SR-RSV-CEF cells were similar to those of the untreated cells. Mn^{2+} stimulated the ectotransferase of the transformed cells more than that of the control CEF cells. Mg^{2+} had a slight stimulatory effect on the transferase of both cell types. Ca^{2+} (8 m*M*) inhibited the activity of the ectotransferase system of CEF cells more than it inhibited catalysis by the transformed cells. Ca^{2+} (0.8 m*M*) had no effect on the activity of either ectotransferase system.

Enzymatic removal of incorporated ^{14}C-sialic acid and pH optima. As indicated in Table II, up to 50% of the radioactivity incorporated onto the surface of either CEF or SR-RSV-CEF cells in the presence of Mn^{2+} could be cleaved by subsequent neuraminidase treatment with 0.04 units for 15 min at 37°C. Longer incubations could increase the amount of radioactivity removed, but cell viability decreased greatly. Figure 1 indicates that incorporation of ^{14}C-NANA into both cell types increased as the pH of the incubation medium was decreased. If the control or transformed cells were incubated with CMP-^{14}C-NANA at a pH below 6.5, the amount of radioactivity which was subsequently removable with neuraminidase decreased. The exact cause of this phenomenon was undetermined, but since it was possible that structures other than the cell surface were increasingly labeled below pH 6.5, all ectotransferase assays were incubated at pH 6.5 or above. No distinct pH optimum above pH 6 was detected for either transformed or control ectotransferase system activity (Fig. 1). This may be due to the difficulty in selectively labeling the cell surface below pH 6.5.

TABLE II. THE INFLUENCE OF NEURAMINIDASE AND VARIOUS CATIONS ON SIALYL ECTOTRANSFERASE SYSTEM ACTIVITY AND REMOVAL OF INCORPORATED ^{14}C-SIALIC ACID BY NEURAMINIDASE.[a]

System	Cell line	
	CEF	SR-RSV-CEF
Intact cells + CMP-^{14}C-NANA	119 ± 15	96 ± 8
Neuraminidase-treated intact cells + CMP-^{14}C-NANA	302 ± 50	235 ± 40
Neuraminidase-treated intact cells + CMP-^{14}C-NANA + 8 m*M* $MnCl_2$	438 ± 40	411 ± 50
Neuraminidase-treated intact cells + CMP-^{14}C-NANA + 8 m*M* $MgCl_2$	311 ± 20	256 ± 15
Neuraminidase-treated intact cells + CMP-^{14}C-NANA + 8 m*M* $CaCl_2$	231 ± 38	219 ± 40
Neuraminidase-treated cells labeled with CMP-^{14}C-NANA + 8 m*M* $MnCl_2$, then washed twice and reincubated with neuraminidase	193 ± 18	188 ± 20

[a] Experiments using intact or neuraminidase-treated cells were performed at pH 6.5 as described in Experimental Procedures. All data are cpm per mg of protein and are means ± 1 SD of experiments on five separate cell cultures.

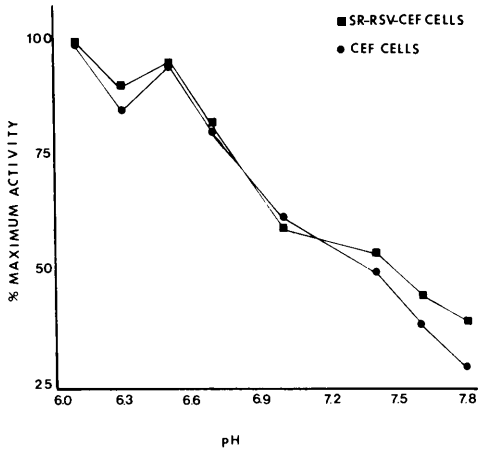

FIG. 1. Effect of varying the reaction pH on the activity of the sialyl ectotransferase system of neuraminidase-treated cells. Data are means of three experiments performed as described in Methods.

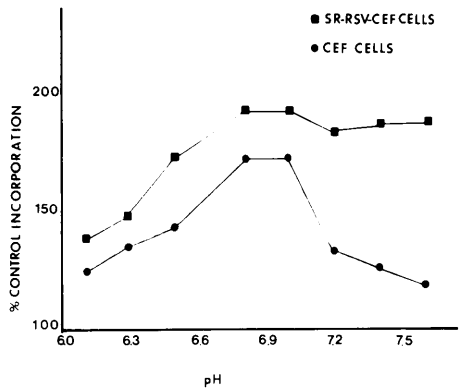

FIG. 2. Stimulation by Mn^{2+} of the sialyl ectotransferase system activity of neuraminidase-treated cells. Control cells were treated with neuraminidase and incubated with CMP-^{14}C-NANA without Mn^{2+}. Experiments performed as given in Methods. Data are means of three experiments.

Variability of Mn^{2+} stimulation with pH. Figure 2 illustrates the quantitative difference in Mn^{2+} stimulation of the CEF and SR-RSV-CEF ectotransferase systems as a function of pH. The sialyl ectotransferase system of the transformed cells is more stimulated by the presence of Mn^{2+} ions over the entire pH range studied (6.0–7.8). The difference between the CEF cells and SR-RSV-CEF cells is more pronounced above pH 7.0 where the control cell enzyme system is only slightly stimulated by the addition of Mn^{2+} ions.

Effect of temperature and time of incubation. The activity of both the CEF and SR-RSV-CEF ectotransferase systems was optimum at 40°C (Fig. 3). It has been demonstrated that when the control and transformed CEF cells are maintained at 41°C, they grow faster and have higher levels of ectotransferase activity (3). Under the optimum conditions of pH and ion concentration, the activity of the CEF and SR-RSV-CEF ectotransferases increased linearly with time up to 15 min, when enzyme activity began to plateau. Ectotransferase system assays were, therefore, terminated at 15 min.

Discussion. The results presented here show that intact CEF and SR-RSV-CEF cells are capable of the enzymatic transfer of sialic acid from CMP-NANA onto endogenous surface acceptors which are increased by

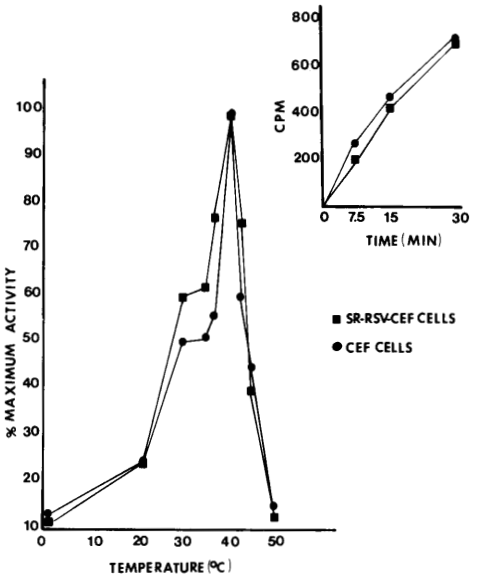

FIG. 3. Effect of temperature and time on the activity of the sialyl ectotransferase system of neuraminidase-treated cells in the presence of 8 m*M* Mn^{2+}. Experiments were performed as described in Methods.

prior treatment of the cells with neuraminidase. The enzymatic nature of this phenomenon is indicated by its linearity with time and its temperature dependence. Location of sialic acid acceptors and transferases on the cell surface is justified by the increase in ectotransferase system activity with neuraminidase and the subsequent removal of ^{14}C-sialic acid with neuraminidase. Similar increases in activity of ectotransferase systems after neuraminidase have been demonstrated by Bernacki (2) in L1210 leukemic cells and by Dalta (4) in mouse fibroblasts.

The data suggest that, under the conditions of assay described here, incorporation of sialic acid from CMP-NANA onto whole cell surface acceptors is altered in transformed cells (Table II). However, activity of the ectotransferase assay system could be limited by the availability of endogenous surface acceptors. Conclusions about absolute enzyme levels are, therefore, not valid. Dalta (4) has recently shown that the ectotransferase activity of a transformed mouse fibroblast line (Ts-3-BHK clone 7C) is increased when the cells are grown at a temperature that does not permit transformation. The CEF and SR-RSV-CEF cells studied here exhibited sensitivities to cation additions which suggests that a different surface phenomenon is being studied after transformation.

Evidence exists for the presence of two glycosyl transferase systems in the livers of virus-infected animals (11), and for slightly different mannosyl transferase systems in normal and arbovirus-infected cells (12). Possibly the ectotransferase system is altered during transformation or more than one sialyl transferase might be present on the surface of transformed cells. If this were the case, the altered ectotransferases on the surface of transformed cells might be an explanation for differences in cell to cell adhesion.

It is clear from the data presented that neuraminidase treatment of intact cells increases the activity of the ectotransferase system presumably by increasing cell surface acceptor availability. Recently Lloyd and Cook (13) have suggested that neuraminidase increases cellular aggregation by generating acceptor sites for cell surface glycosyltransferases. The percentage increase in ectotransferase system activity after neuraminidase was the same for control and transformed CEF cells, indicating that acceptor availability is not different in the two systems. The possibility that altered glycosyltransferase acceptors are present on the surface of transformed cells cannot be eliminated.

Finally, the results of these studies make clear that in any assessment of cell-surface transferase system activity, the cell's environment, including pH, ion concentration, temperature, and cell density must be carefully controlled. It is possible by manipulating the conditions of assay to have higher levels of ectoenzyme activity in the transformed cells, as shown previously (3), or lower, as presented here. The fact that *in vitro* the sensitivity of the ectoenzyme system to ion addition varied with pH, particularly in the case of control CEF cells, may mean that *in vivo* similar changes in the cell's environment could modulate surface ectotransferase activity and thereby influence such phenomena as contact inhibition.

Summary. The activity of the sialyl ecto-

transferase system of normal chick embryo fibroblasts (CEF) and chick embryo fibroblasts transformed with the Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV) have been compared. Neuraminidase treatment of the intact cells increased the sialyl ectotransferase system activity of control and transformed cells two to three times. The ectotransferase system activity increased as the pH was decreased from 7.8 to 6.0. The temperature optimum for both systems was 40°C. Approximately 60% of the ^{14}C-sialic acid incorporated at pH 6.5 or above could be removed with neuraminidase. The activity of the transformed cell system with or without neuraminidase treatment was more stimulated by addition of Mn^{2+} ions, particularly above pH 7.0. This difference in ion sensitivity indicates that a different cell surface phenomenon is being studied after transformation.

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