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DIFFERENCES IN SIALIC ACID CONTENTS OF LOW CANCER CELLS, HIGH CANCER CELLS AND NORMAL MOUSE LUNG COUNTERPARTS

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SUMMARY

Sialic acid contents of low cancer (P 4 BIS) high cancer (P 4 BIS T) cells and their normal (PB) mouse lung counterparts have been determined. This content is 5 to 10 fold higher for cells in logarithmic phase growth than for confluent cells, as well for normal cells as for transformed derived cells lines. Growing normal PB cells contain a large amount of sialic acid (21.2 $\mu g/10^6$cells) : it is reported that cellular sialic acid content decreases dramatically with the tumor producing capacities of the cells (3.4 $\mu g/10^6$ P 4 BIS cells ; 2.1 $\mu g/10^6$ P 4 BIS T cells).

It has been found in conditions which maintain cell viability that transformed neuraminidase treated cells or trypsin treated cells liberate large percentages of sialic acid, or sialoglycoproteins, whereas small percentages are liberated from normal cells, indicating that transformed cell surface glycoproteins may be reached more easy by enzymes that normal cells : in that aspect low cancer cells (P 4 BIS) appear transitory between normal (PB) and high cancer cells (P 4 BIS T) in the same way they are transitory in tumor producing capacities.

INTRODUCTION

Many of the studies directed towards understanding the mechanism of transformation and tumorigenesis have focused on comparisons between normal and transformed cells and have revealed a number of transformation associated properties (1,2). It has been reported that cell surface glycoconjugates are implicated in cells functions(3) this data being also supported by use of lectins which bind specifically the glycoconjugates (4, 5, 6).

Among the different sugar residues present in the mammalian glycoproteins, sialic acid seems to have a particular role on biological function as it is located on the external cell membrane surface (7, 8, 9).

Concerning the sialic acid content of malignant cells and normal counterpart contradictory results have been reported (9) indicating that there is

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no direct change of the sialic acid content upon transformation. In some cases malignancy is associated with decreased level of sialic acid (10) and in other cases with and increased glycosylation and consequently of sialic acid content (1, 11, 12, 13).

This paper reports the comparative sialic acid content for normal mouse lung cells and their low cancer and high cancer derived cell lines with the purpose of correlating the sialic acid content with the cells tumorigenicity degree.

EXPERIMENTAL PROCEDURE

- Cell culture. Normal PB cells were prepared from 3 week old females C 57/Bl 6 mouse lung - Centre d'élevage CNRS - ORLEANS - FRANCE - grown in vitro and used up to their 6 th passage.

Cancer cells p 4 BIS and P 4 BIS T were permanent cell lines of the same origin (i.e C 57/Bl 6 mouse lung cells) whose differences in biological properties and tumor producing capacities have been described earlier ; P 4 BIS have been described as low cancer cells and P 4 BIS T as high cancer cells. The tumor producing capacity expressed as TD 50 is 10 fold higher for P 4 BIS T cells than for P 4 BIS cells (14).

Cell cultivation were performed in Eagle minimum essential medium (MEM) supplemented with 10% foetal calf serum (FCS) aminoacid (4X) ; penicillin (100 U) and streptomycin (100 U) were added just before use.

Subcultivations were performed after dispersion by 0.25% trypsin for P 4 BIS T cells and 2,5% trypsin for PB and P 4 BIS cells ; for further biochemical experiments the cell monolayer were washed three times with PBS and harvested with 2 mM EDTA in PBS, (phosphate buffered saline) with proteinase inhibitor (2 mM PMSF : phenylmethylsulfonylfluoride).

- Cell viability tests. The cell viability remaining after the different biochemical treatments was tested either by trypan blue exclusion or platting efficiency (15).

- Growth of cells. The time course of growing cells has been determined by spreading the cells into Lindbro multiwells and by counting the cells grown after 1 to 72 hours, without removing the medium. The spread cells suspension was in each case of 5.10^4 cells/ml.

- Trypsin treatment. Before the trypsin treatment subconfluent cells were grown 24 hours in FCS - free medium. They were washed three times with PBS and cells monolayers treated with TPCK - Trypsin (3 µg/ml) at 37° as long as cells viability remains (5 to 30 minutes). The optimal conditions for cell membrane glycoconjugate liberation were determined by testing the cells viability after different periods of treatment.

- Neuraminidase treatment. Before neuraminidase treatment the three subconfluent cell types were grown 24 hours in FCS - free medium. Cells were washed twice with PBS pH 7.4 and twice again with pH 7 $NaHCO_3$ buffered 0.15M Nacl. Neuraminidase treatment was performed in the same buffer pH 7 with 50 units enzyme/ml, as long as cells remain alive, after being removed from the plastic bottle by the enzymic treatment.

- Total cell sialic acid content. PB, P 4 BIS and P 4 BIS T cells grown 24 hours in FCS - free medium were washed three times with PBS and treated at 37°C with 2 mM EDTA until they were removed from the plastic bottle.

The time depends on the type of cell and varies from 5 to 20 minutes. Isolated cells, EDTA supernatant and plastic adhesive molecules were treated for sialic acid determination.

- Sialic acid determination. Sialic acid was determined using the thiobarbituric assay (16) either after neuraminidase treatment or 0,01 N HCl

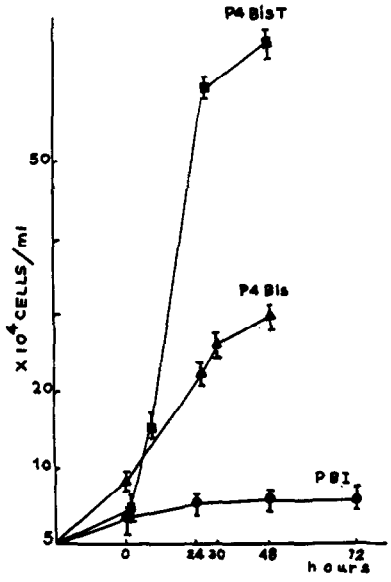

Fig. 1 : Time course of P B, P 4 BIS et P 4 BIS T growing cells determined by counting the cell grown after 1 to 72 hours without removing the medium, after initial spreading of 5.10 h cells/ml.

hydrolysis at 80° for 1 hour. Complementary determination of sialic acid has been performed by chromatography.

- Specific labelling of cell surface sialyl residues. Sialyl residues from the cell surfaces were labelled according to a procedure derived from that reported by Dodeur and Bourrillon (17). Cell monolayers were washed 3 times with p H 7.4 $NaHCO_3$ buffered - 0.15 M Nacl, and treated with 10^4 M Na meta-periodate for 10 minutes. Reduction with NaB^3H_4 (approxiatively 10^5 cpm for 10^6 cells) was performed for 8 minutes. Labelled cells were washed several times, harvested with 2 mM EDTA and used for further investigation after 3 washes with PBS pH 7.4.

Comparative cells in suspension were labelled on their sialyl residues by the same chemical procedure : cell suspensions were obtained by scraping cells from the flask. In that condition, the entire surface of the cell membrane should be labelled, and not only the upper side of the monolayer.

RESULTS

- Time course of growing cells - The time course of PB, P 4 BIS and P 4 BIS T growing cells are reported fig.1. The logarithmic phase of growth and the plateau phase were determined for each cell type, after initial spreading of 5.10^4 cells/ml (3 ml/25mm diameter plastic dish.)

- Sialic acid of whole cells -The results obtained for either PB, P 4 BIS or P 4 BIS T cells are dramatically different if one considers sialic acid liberated from exponential phase growing cells and stationary phase growing cells (Table II).

TABLE I

Sialic acid of normal PB, low cancer P4 BIS and high cancer P 4 BIS T murine cells.

	PB	P_4BIS	P_4BIS T
Total cell sialic acid	21,2	3,4	2,1
Sialic acid liberated from neuraminidase treated cells	6,5	2,5	1,6
Sialic acid content of trypsinisates	1,5	1,5	1,7
% of cell sialic acid liberated by neuraminidase	30,8%	73,5%	76,2%
% of cell sialic acid present in the trypsinisate	7 %	44 %	81 %
Alive cells NaB^3H_4 external sialic acid labelling (cpm/10^6 cells)	5200	3050	1890
Trypsinisate radioactivity of labelled cells (cpm/10^6 cells)	1500	1350	1700

Results are expressed in µg of sialic acid on the basis of 10^6 cells of each type. These values are the mean of 3 to 8 determinations performed on 10^7 to 4.10^8 cells.

It appears that confluent cells (stationary phase) exhibit a sialic acid content 5 to 10 fold lower that the exponential phase growing cells as well for normal PB cells as for P 4 BIS and P 4 BIS T malignant cells (Table II).

The quantity of sialic acid of whole cells decreases with the appearance and evolution of the tumorigenic potentialities of the malignant cells.

- Neuraminidase treatment - Neuraminidase treatment of viable cells has been performed on monolayer as cells remain alive longer than after scraping : furthermore scraped cells have tendancy to aggregate and the neuraminidase treatment performed on cells suspension obtained after scraping release only 10 to 30 % of the sialic acid liberated by neuraminidase treatment of monolayer.

In that condition it has been determined that most of the sialic acid accessible to neuraminidase is liberated in the first 15 minutes of treatment and that cell aggregation is partially due to almost sialic acid-free cells.

The maximal quantity of sialic acid liberated by neuraminidase from PB, P 4 BIS and P 4 BIS T cells reported table II, has been determined on exponential phase growing cells ; in standardized conditions cells were treated as

TABLE II

Comparative sialic acid contents of PB, P 4 BIS and P 4 BIS T murine lung cells, in logarithmic phase and stationary phase of growth.

	PB	P 4 BIS	P 4 BIS T
Logarithmic phase of growth	21.2	3.4	2.1
Confluency (stationary phase of growth)	2.50	0.56	0.25
Ration of sialic acid from stationary phase of growth to exponential phase.	11.7 %	16.4 %	11.9 %

Results are expressed in µg of sialic acid per 10^6 cells.
These values are the mean of 3 to 6 determinations.

long as they remain alive after being liberated from the bottle. This corresponds to 10 to 20 minutes treatment and to the maximal sialic acid liberated.

- Sialic acid content of cell trypsinisates - The sialic acid content of trypsinisate of live PB, P 4 BIS and P 4 BIS T cells has been determined and reported Table I.

Comparatively the amount of sialic acid liberated by trypsin from 10^6 cells are similar in normal and transformed cells, but however these quantities correspond to a small amount of the total sialic acid for normal PB cells (7%) and a greater ratio for transformed P 4 BIS (44%) and P 4 BIS T cells (81%) is observed.

DISCUSSION

Our results obtained for the sialic acid content of low cancer (P 4 BIS), high cancer (P 4 BIS T) cells and their normal counterparts (PB) show that normal mouse lung cells contain a large amount of sialic acid residues per cell as compared to transformed ones.

These results are consistent with those reported for virally transformed 3T3 cells in culture which possess less sialic acid than their normal counterpart (18, 19, 20) and inconsistent with other and principaly those of Warren et al (1, 11, 12, 13).

In our mouse lung system, normal cells sialic acid content is 10 fold higher than the transformed cells one : such a discrepancy between normal and transformed cells has not been reported, and to our knowledge the largest

difference to be published was by Makita and Seyama which reported that plasma membrane from transformed PY BHK cells had 60% less sialic acid than membrane from normal BHK cells (20).

Several possibilities non exclusive one from another which can explain those results have been investigated and reported elsewhere.

i) The small amount of sialic acid residues of transformed cells may be due to a general decrease of total glycoproteins quantity associated with transformation. Some experiments now in progress on this mouse lung system, using sugar endogeneous labelling of glycoproteins with radioactive sugar seem to be in agreement with this possibility, which appears the most probable.

ii) It has been reported that transformed cells in culture contain less sialyltransferase activity than normal cells (20) and consequently the biosynthesis of the glycoprotein is incomplete giving rise to partially asialoglycoconjugates (22, 23). This explanation may be forwarded as contradictory results has been reported (12).

iii) An other explanation emerges if one considers the glycolipids of the cell membrane. It has been described that normal cells in culture contain more sialoglycolipids than transformed cells, and glycolipids from transformed cells exhibit deletion of carbohydrates(24).

In the same point of view the presence of sialidase in plasma membrane has been reported (25) and it has been demonstrated that sialidase activity towards gangliosides is only observed for transformed cells and not for normal ones (25).

It has been demonstrated in mammalian cells that 70% of the sialic acid residues are located on the plasma membrane ; 80% of the latter being able to be liberated from live cells by neuraminidase, however sialic acid is removed more easily by neuraminidase from transformed cells than from normal cells (19). Our values are consistent with this result as 31% of the total sialic acid is removed by neuraminidase from live PB cells and up to 75% from live transformed cells, low cancer cells and high cancer cells having almost the same susceptibility towards neuraminidase as long as they remain alive.

The determined specific radioactivity of labelled sialic acid of 1000 cpm/µg of sialic acid. Our results (Table II) clearly show that the quantity of sialic acid removed from live cells by neuraminidase treatment corresponds to the quantity of external sialic acid specifically labelled. This quantity is somewhat lower for normal cells than for transformed cells, indicating that sialic acid of normal PB cells may be masked for chemical labelling and neuraminidase accessibility. In the same way sialoglycoprotein liberation from live cells by trypsin is also proportionnally higher for transformed cells

than for normal ones, high cancer live cells being able to liberate almost their total sialic acid content in the sialoglycoprotein split by trypsin. This indicates that much of the sialic acid of normal live cells may be masked even if they are located at the external cells surface.

It is well known that macroglycoproteins from cells surface play an important role in cells social behaviour, and particularly the sialic acid residues (27), and it was interesting to compare the cells sialic acid content during logarithmic phase of growth and confluency. In all cases the content determined for confluent cells corresponds to 10-12% of the amount found for logarithmic phase, whatever the degree of tumorigenicity of cells.

Similar findings have been reported, indicating that there is an inverse correlation between the sialic acid content and saturation density of various cells lines (27). Concurrently Warren et al have demonstrated that growing cells have more of specific sialyl transferase than cells from confluent cultures (23). Since contact-inhibited cells grow to low saturation densities, these results suggest some relationship between sialic acid levels and social behaviour. In addition Buck et al (28) reported, that the differences between cells surface glycoproteins from normal and transformed cells are more evident using logarithmic phase growing cells.

In conclusion it is tempting to connect changes in sialic acid with expression of the transformed state of cells, and consequently with social behaviour, but many unexplained discrepancies prevent this conclusion.

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