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Regeneration of the Surface Glycoproteins of a Transplantable Mouse Tumor Cell After Treatment With Neuraminidase

(sialic acid/cytotoxic factor)

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ABSTRACT **Strain A mouse ascites tumor cells that were treated with neuraminidase (EC 3.2.1.18) (these cells show decreased tumor-forming ability in allogeneic C3H mice) rapidly regenerate sialoglycoproteins at the cell surface during culture. The incorporation of labeled D-glucosamine into membrane glycoproteins of cells that were treated or untreated with neuraminidase proceeds at similar rates. Surface glycoproteins that contain sialic acid are synthesized *de novo* during culture of neuraminidase-treated cells, and in nondividing cells, synthesis is accompanied by turnover of the membrane glycoproteins. The rate of turnover of membrane glycoproteins that lack sialic acid residues is the same as that occurring in nondividing cells that are not treated with neuraminidase and that are cultured under identical conditions. Turnover of the surface membrane of nondividing cells leads to the accumulation of glycoproteins in the supernatant medium of cell cultures. The rapid regeneration of cell surfaces that contain sialic acid by TA3 cells that were treated with neuraminidase, makes it unlikely that the rejection of these cells *in vivo*, in C3H mice, is due solely to the induction of a primary immune response to new or to previously concealed antigenic specificities that would be expressed on the surface of the sialic acid-depleted cell.**

The TA3 tumor cell, derived from a spontaneous mammary adenocarcinoma of an A strain mouse, has a surface membrane that is rich in carbohydrate material (1, 2). The importance of the surface glycoproteins in tumorigenesis has been emphasized (3, 4). Some sublines of the TA3 tumor cells have lost strain specificity during subculture and are transplantable in certain strains of allogeneic mice for reasons that are still not clear. Sanford (5) showed that transplantability of TA3 tumor cells in allogeneic C3H mice was markedly reduced by neuraminidase (EC 3.2.1.18) treatment and suggested that this could be due to some immunological response of the host to altered TA3 cell membranes. This result was unexpected in view of the rather rapid regeneration of the surface coat observed histochemically (3). We have reexamined the process of regeneration of the surface glycoproteins of cells that were treated with neuraminidase and confirmed that the cells quickly revert in cell culture to a more normal surface.

MATERIALS AND METHODS

Ascitic fluid from strain A mice (West Seneca Laboratory of Roswell Park Memorial Institute, Buffalo, N.Y.) was withdrawn aseptically 7 days after the mice were inoculated with about 50,000 TA3 cells per mouse. The cells (up to 2×10^8 cells per mouse) were washed several times with a balanced salts solution and, when required, treated with *Vibrio cholerae* neuraminidase (Behringwerke, West Germany, American Hoechst Corp., Woodbury, N.Y.) for 1 hr (2) at 24° for removal of at least 80% of the total sialic acid present in the cells (2). Control cells were kept in a balanced salts solution for 1 hr at 24°. The cells were dispensed at about 10^6 cells per ml into tissue culture dishes (Falcon Plastics Corp., Los Angeles, Calif.) containing medium 199 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with fetal calf serum (10%)–penicillin (100 units/ml)–streptomycin (100 μg/ml). The calf serum was heated for 30 min at 56° before use, since earlier work (4) suggested the presence, in certain batches, of a factor cytotoxic for cells that were treated with neuraminidase. The cell suspension culture was incubated at 37°.

The incorporation of D-[^{3}H]glucosamine into glycoproteins was measured as follows: TA3 cells and TA3 cells that were treated with neuraminidase were cultured in a medium containing D-[^{3}H]glucosamine (10 Ci/mol, 4 μCi/ml). At times, duplicate cultures, each containing 5 ml, were removed, and the washed cells were treated with cold 5% (w/v) trichloroacetic acid (1 ml) overnight. Insoluble fractions were washed with cold trichloroacetic acid, solubilized in hyamine (1 ml), and counted in a liquifluor–toluene mixture. The acid-soluble fractions were freeze-dried, dissolved in water, and counted in a *p*-dioxane-based scintillation fluid. Washed cells were also suspended in a balanced salt solution (0.5 ml) and treated with trypsin (Worthington Biochem. Corp., Freehold, N.J., two times crystallized, 50 μl of solution, 0.5 mg/ml) for 30 min at 24°. The soluble materials released by trypsin were obtained in the supernatants after centrifugation and passed through a column of Sephadex G-15. The glycoprotein fractions, eluted in the void volume of the columns, were freeze-dried and dissolved in water, and the radioactivity was counted.

The nucleic acid and protein syntheses were measured in cells cultured in media containing either [2-^{14}C]uridine (0.4 μCi/ml, 54 Ci/mol), [4,5-^{3}H]leucine (0.4 μCi/ml, 5 Ci/mmol) or [*methyl*-^{14}C]thymidine (0.4 μCi/ml, 55 Ci/mol). Trichloroacetic acid precipitates were prepared and counted as described.

Chromatography on columns (2×30 cm and 2×60 cm, respectively) of Sephadex G-15 and G-150 was performed in

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0.1 M ammonium acetate, pH 5.5. Fractions (1.5 ml) were collected and appropriate tubes were combined and freeze-dried for removal of the eluting buffer salts.

Neuraminidase-treated or untreated TA3 cells were used as indicator cells in cytotoxicity tests, in microtiter plates. Guinea-pig serum, at several dilutions, and target cells were mixed and kept for 30 min at 37° before centrifugation. Dead cells were estimated by measurement of the ^{51}Cr released or by staining with Trypan Blue. Guinea-pig serum, preheated for 30 min at 56°, was used as a control, and in all cases, the serum did not lyse either of the target cells (cells that were treated or untreated with neuraminidase).

Sialic acid residues were removed from glycoprotein material by mild hydrolysis for 1 hr at 80° in 0.1 M HCl. Sialic acid was isolated from the dried hydrolysates by paper electrophoresis in a buffer of pyridine–acetic acid–water, 6:46:323 (v/v/v) (pH 3.6) at 1000 V for 1–2 hr. The identity of the sialic acid was controlled by paper chromatography in butyl alcohol–propyl alcohol–0.1 M HCl 1:2:1. Glucosamine and galactosamine were isolated from the glycoproteins after hydrolysis in 4 M HCl for 6 hr at 100° by preparative paper chromatography in pyridine–ethyl acetate–acetic acid–water 5:5:1:3 (by volume).

RESULTS

Treatment of TA3 cells with neuraminidase had no effect on growth in culture. The cell number increased less than 15% during the first 15–25 hr of culture. This was followed by a burst of cell division over the next 15–25 hr leading to about 2- to 3-fold increase in cell number. Thereafter, the cultures remained at constant density for at least 72 hr without the addition of fresh medium. Cell viability remained high (greater than 90%). In most incorporation experiments, we have used cells that are in the earliest part of the growth curve, i.e, cells apparently not undergoing extensive cell division.

Synthesis of macromolecules in TA3 cells treated with neuraminidase was very similar to that in control TA3 cells, as judged by rates of incorporation of labeled thymidine, uridine, and leucine into DNA, RNA, and protein fractions precipitable by cold, dilute trichloroacetic acid, respectively.

Neuraminidase treatment also had no effect on the capacity of TA3 cells to use D-[^{3}H]glucosamine and incorporate it into cellular glycoproteins (Fig. 1). In both cells that were treated or untreated with neuraminidase there was an approximately linear incorporation of glucosamine, for at least 20 hr, into acid-precipitable material. The rate of incorporation of glucosamine into glycoproteins released from the surface of intact cells with trypsin, which is a measure of the membrane-glycoprotein synthesis, was found to be approximately linear (Fig. 1). It had been shown (2) that trypsin removes up to 75% of the sialic acid residues from the surfaces of intact cells in the form of glycoproteins or glycopeptides of relatively high molecular weight. About 40% of the total acid-precipitable counts were present in the trypsin-soluble fraction (Fig. 1).

Labeled glycoprotein released by trypsin from the surface of intact TA3 cells cultured for 5 hr in a medium that contained radioactive glucosamine was isolated in the excluded volume, after chromatography on Sephadex G-15. Acid hydrolysis followed by quantitative isolation of the constituent sugars by paper chromatography showed that the radioactivity was present in glucosamine (46%), galactosamine (29%), and sialic acid (25%). More than 85% of the total

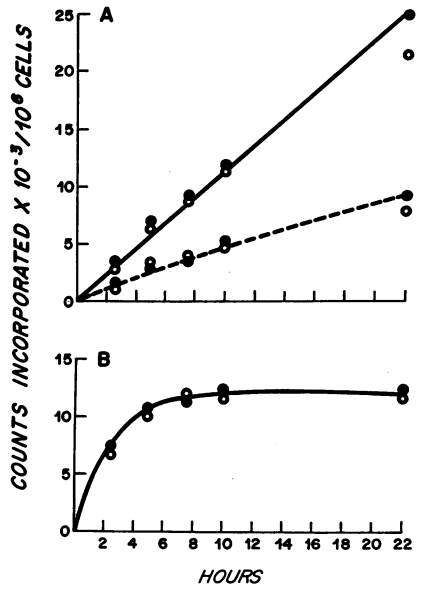

FIG. 1. Time course of incorporation of D-[^{3}H]glucosamine into intact cultured TA3 cells (●) and cells treated with neuraminidase (○). (*A*) Rate of incorporation into trichloroacetic acid-insoluble fractions of whole cells (——) and into the fractions released from intact cells by treatment with trypsin (– – –). (*B*) Incorporation of D-[^{3}H]glucosamine into trichloroacetic acid-soluble fractions of intact control cells (●) and of intact cells treated with neuraminidase (○).

radioactivity present in the glycoprotein fraction was recovered. After a similar incubation of TA3 cells that were treated with neuraminidase, the distribution of radioactivity in glucosamine, galactosamine, and sialic acid was 48, 30, and 22%, respectively. Similar results were obtained for the distribution of radioactivity in the monosaccharides isolated from trypsin-soluble fractions that were prepared from TA3 cells incubated for 46 hr in a medium containing D-[^{3}H]glucosamine.

The incorporation shown in Fig. 1 apparently took place with a less than 10% increase in cell number and no noticeable increase in cell size. Therefore, it seemed likely that the incorporation observed in the cells that were not growing was due to turnover of the surface membranes (6). This was shown to be the case by the pulse-chase experiment illustrated in Fig. 2. In the absence of turnover, the radioactivity obtained in the soluble fractions released by trypsin from cells in 5 ml of culture would remain constant during the chase. This was found to be approximately so (Fig. 2*A*) in cells growing exponentially in a nonradioactive medium, after a 25-hr incubation in a medium that contained D-[^{3}H]glucosamine.

The amount of radioactivity found in fractions of cells that were not growing (Fig. 2B) decreased markedly during the period of chase. No appreciable difference was found in the rate of decay of radioactivity in the trypsin-sensitive glycoproteins present at the surface of TA3 cells that were treated with neuraminidase, as compared to the control TA3 cells. It was not possible to extend the chase period beyond 27 hr, since the cells then began actively to divide. During the chase period, radioactivity was found (Fig. 2*B*) in the culture fluids remaining after removal of the cells by centrifugation. The bulk of the radioactivity present in culture fluids of cells chased for 6 and 21 hr was found in material that was eluted unretarded from Sephadex G-150 as a single, sharp peak that

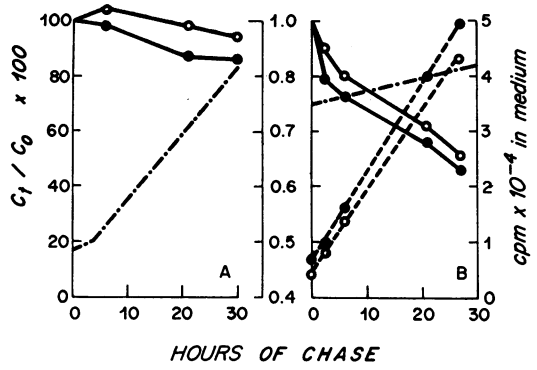

FIG. 2. Influence of prior treatment with neuraminidase on the turnover of surface glycoproteins in growing cells (*A*) and in cells that were not growing (*B*). (*A*) TA3 cells in the exponential growth stage were kept in a culture medium containing either D-[^{3}H]glucosamine (10 Ci/mol, 4 μCi/ml) or D-[^{14}C]glucosamine (2 Ci/mol, 1 μCi/ml) for 21 hr at 37°. The washed ^{3}H-labeled cells (15×10^6) were treated with neuraminidase, washed twice, and mixed with control ^{14}C-labeled cells (15×10^6). The mixture of cells was then incubated at 37° in a medium that does not have radioactivity (initial cell density 0.66×10^6 cells/ml). Tritium counts (○) indicated the turnover of glycoproteins from cells treated with neuraminidase; carbon-14 counts (●) indicated the turnover of glycoproteins from untreated cells. (*B*) TA3 Cells were labeled *in vivo* in a mouse by intraperitoneal injection of D-[1-^{14}C]glucosamine, and then were removed 24 hr later. Half of the cells (about 1×10^8 cells) were treated with neuraminidase; the treated cells (○) and control cells (●) were then cultured separately (initial cell density 1.2×10^6 cells/ml) in 5-ml portions of a medium that does not have radioactivity. At times, duplicate portions (each 5 ml) of the cultures from *A* or *B* were removed, and the washed cells were treated with trypsin to release the soluble glycoproteins from the surfaces of cells. Radioactivity found in the trypsinates of cells treated with neuraminidase (○) and control cells (●) is expressed as a percentage (C_t/C_o) $\times$ 100 of radioactivity present at the beginning of the chase period. The medium supernatants obtained after centrifugation of the cultures from *B* were also counted (- - - - -). Cellular growth during the chase is indicated by -·-·-; middle ordinate, logarithm of cell number $\times 10^{-6}$ per sample.

emerged immediately behind the void volume. After treatment of the high molecular-weight glycoprotein fraction with trypsin (1 mg/ml) overnight at 24°, its elution profile was very little changed during repeated chromatography on Sephadex G-150.

Recently, a factor cytotoxic towards neuraminidase-treated TA3 cells but not toward untreated TA3 cells was found to be present in C3H mouse serum but was lacking in sera of A, C57BL/10, and DBA/2 mice (4). These strain differences are of particular interest, since Weiss and Cudney (7) have reported that neuraminidase treatment does not alter *in vitro* interactions between C57BL/10 spleen cells and mastocytoma P815 cells, while Sanford and Codington (4) have observed no effect of neuraminidase treatment on transplantability of TA3 cells in DBA/2 hosts. Toxicity for TA3 cells that were treated with neuraminidase was also found in guinea pig serum (4), and we have used this effect to follow the rate at which neuraminidase-treated TA3 cells revert, in culture, to a more normal cell surface.

It will be seen (Fig. 3) that treated TA3 cells rapidly became resistant to the cytotoxic effect of guinea pig serum, with a half-life of 3–4 hr. It is difficult to correlate these findings with the turnover rate of the bulk of the surface glycoproteins of cells that were not growing, determined by the pulse-chase experiment (Fig. 2*B*), since we do not know the content of sialic acid of membrane glycoprotein required to confer resistance to the cytotoxic factor. Furthermore, the receptors present in the membrane of neuraminidase-treated cells for the cytotoxic factor may turn over more rapidly than the bulk glycoprotein fraction released from cells after treatment with trypsin.

DISCUSSION

The rate of turnover of the surface glycoproteins of the TA3 cell reported in this paper is of the same order as that found for L-cells by Warren and Glick (6), which substantiates for the TA3 cell the general conclusions of these authors for the L-cell. The rate of membrane synthesis is apparently about the same for growing cells and for cells that are not growing. In the actively dividing cell, new membrane material is utilized in the expanding cell surface and there is little turnover. In the absence of an increase in cell numbers or cell size, the overproduction of membrane components is compensated for by a relatively high rate of turnover.

The molecular size of the glycoproteins removed from the TA3-cell surface during turnover, as indicated by elution from Sephadex, was not noticeably changed by treatment with trypsin, indicating that the polypeptide moieties were relatively resistant to trypsin. Recently, glycoprotein fractions isolated from the TA3-cell surface have been shown to lack appreciable amounts of basic amino acids (8). The release of surface glycoproteins of high molecular weight into the extracellular medium by TA3 cells during turnover (Fig. 2*B*) may contribute to the ability of these cells to cross major histocompatibility barriers. The substances released from the cell membranes could conceivably act to block allogeneic host lymphocytes after transplantation and enable the tumor cells

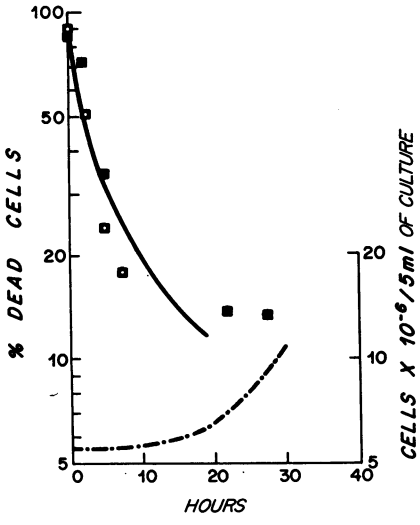

FIG. 3. Development, by neuraminidase-treated TA3 cells, during culture, of resistance to the cytotoxic effect of normal guinea pig serum. At times during cell culture, 5-ml portions were withdrawn, and the washed cells were tested for resistance to lysis by normal guinea pig serum by means of Trypan Blue permeability (□) and ^{51}Cr release (■), as indices of cell lysis. Cellular growth during culture is indicated by -·-·-.

to survive in heterologous hosts (9). Immunological enhancement with soluble histocompatibility antigens of allogeneic tumor growth has been described (10), and these antigens may well be among the components shed by TA3 cells during turnover.

The patterns of incorporation of a D-[^{3}H]glucosamine precursor into the glycoproteins during relatively short pulses by TA3 cells that are not growing and that had been treated or untreated with neuraminidase are very similar. This eliminates the possibility of a transfer of sialic acid residues to the carbohydrate chains that lack these residues after neuraminidase treatment. Clearly, the incorporation observed in these cells represents a *de novo* synthesis of complete carbohydrate chains. In contrast, liver plasma membranes show a limited ability to transfer sialic acid residues to endogenous asialoglycoproteins present in the membrane (11).

The restoration of sialic acid residues at the surface of TA3 cells seems to be sufficient to confer resistance to the cytotoxic factor present in normal guinea pig serum. It is likely that the same degree of resistance is conferred to these cells towards the factor present in C3H mouse serum, although this has not been investigated. It is of interest that the rate at which TA3 cells that are treated with neuraminidase become resistant to the factor in normal guinea-pig serum is similar to the rate of resynthesis of viral receptor sites in HeLa cells (12) and of H-2 antigens in Meth A tumor cells (13), after treatment of the cells with proteases.

Since TA3 cells that were treated with neuraminidase were found to survive in culture and to incorporate precursors into nucleic acids and glycoproteins as well as do control TA3 cells, the suggestion (14) that increased resistance of allogeneic hosts may reflect cell damage during neuraminidase treatment is ruled out. The rapid synthesis of fully sialylated glycoproteins at the surface of treated TA3 cells also makes it unlikely that the rejection of these treated cells by allogeneic C3H mice was due to induction of a primary immunological response of the mice to the modified TA3 cells. The probable biological effects of neuraminidase on the immunogenicity of the cells (15, 16) and on blastic transformation of host lymphocytes (17) would lead to consequences that are expressed after a return of the surface of most of the injected TA3 cells previously treated with neuraminidase to an environment that is rich in sialic acid. On the other hand, in spite of the relatively rapid regenerative process observed during culture of treated TA3 cells that leads to a more normal cell surface, a large proportion of the treated TA3 cells would be removed after transplantation by the cytotoxic factor that already existed in C3H mouse serum and that acted even more rapidly than the rate of turnover of the surface glycoproteins. This reduction in the number of tumor cells may be sufficient to allow the challenge to be manageable by the immune response of the host animal to the TA3 cell, resulting in an impaired capacity of the TA3 cells that were treated with neuraminidase to develop tumors successfully in the C3H hosts.

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