Differential Release of Sialic Acid from Normal and Malignant Cells by *Vibrio cholerae* Neuraminidase or Influenza Virus Neuraminidase

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SUMMARY

A comparison was made between the amounts of sialic acid released from viable cell suspensions of normal and malignant cells by *Vibrio cholerae* neuraminidase (VCN) and by influenza virus neuraminidase. More sialic acid can be removed by enzyme digestion from various tumors than from normal lymphoid cells. More sialic acid can be removed from the surface of both normal and tumor cells by VCN than by influenza virus neuraminidase. VCN lyases the 2-3, 2-6, and 2-8 glycosidic linkages between sialic acid and the mucopolysaccharides of the cell surface, and influenza virus neuraminidase lyzes only the 2-3 and 2-8 linkages. Therefore, it appears that most cell-surface sialic acid residues are bound by the 2-6 linkage on both normal and malignant cells. The relative increase in cell-surface sialic acid residues may be important to tumor immunotherapy, since the removal of cell-surface sialic acid residues with VCN increases the immunogenicity of tumor cells.

INTRODUCTION

A number of laboratories have shown that treatment of viable cells with VCN renders them increasingly immunogenic (3-7, 10-13, 32, 35-40). Both normal (19, 35, 36) and tumor (4-7, 10-13, 32, 36, 37, 39, 40) cells are rendered more immunogenic by VCN treatment. Tumors treated with VCN in vitro fail to grow in normally susceptible recipients (4-7, 10-13, 32, 36, 37, 39, 40), and cell recipients are rendered immune to subsequent challenge with normal tumor cells (37-40). Earlier, Currie and Bagshawe (12) and, recently, Bekesi et al. (6) suggested that neuraminidases from sources other than *V. cholerae* did not eliminate the oncogenicity of leukemia L1210 cells, while VCN did. Similarly, we have shown that VCN induces profound alterations in the serological properties of lymphoid cells (19, 26-30) but that IVN has no such effect (29). These 2 enzymes differ in their substrate specificities (14, 15) so that their differential effect can be used as a probe to investigate the binding linkages of the enzyme-susceptible sialic acid in intact cells. In our studies, we attempted to compare the quantities of sialic acid that can be released by VCN or IVN from normal and malignant mouse cells.

MATERIALS AND METHODS

Animals. Inbred mice from The Jackson Laboratory, Bar Harbor, Maine, were used for this study. Most of the tumors also were obtained from The Jackson Laboratory and were maintained in our laboratory by serial syngeneic transplantation. Several methylcholanthrene tumors have been induced in our laboratories (36, 37, 39, 40). All tumors were serially transplanted in syngeneic hosts and were used for sialic acid assay 2 to 4 weeks after transfer. We used C3H/HeJ mice and adult male New Zealand white rabbits to obtain normal lymph nodes, thymus, spleen, bone marrow, and red blood cells.

Collection and Preparation of Cell Suspensions. Mouse or rabbit lymph node, spleen, thymus, femoral bone marrow, and tumors were removed from animals killed by cervical compression. Tumors with necrotic areas were not utilized. We prepared cell suspensions of normal tissue by teasing apart the respective tissues in BSS at pH 7.0 (16), without any indicator. To prepare suspensions of tumor cells, we pressed small pieces of tumors through stainless steel screen (40 mesh) in BSS without the addition of trypsin. Cell clumps were allowed to settle, and the supernatants containing suspensions of single cells were retained and washed 3 times. Cell viability was measured by trypan blue exclusion (8), and only suspensions having greater than 90% viability were used.

Incubation of Cells with Neuraminidase. Neuraminidase from *V. cholerae* (VCN) and from influenza virus B (Lee strain) (IVN) was obtained from General Biochemicals, Inc., Chagrin Falls, Ohio. The former material contained 500 units/ml, while the latter contained 100 units/ml. One unit of VCN activity is defined by the manufacturer as being equivalent to the release of 1 µg of N-acetylmuramic acid from a glycoprotein substrate at 37° in 15 min at pH 5.5. One unit of IVN causes the release of 1 µmole of sialic acid per min at 37° from bovine submaxillary mucin. The enzymes were diluted to appropriate activity concentrations in BSS, mixed with an equal volume (1 ml) of cell suspension, and incubated at 37° for 1 hr. All of the tubes were mixed every 15 min. After incubation, the cells were centrifuged (800 X g for 5 min), and the supernatants were saved for the sialic acid assay.
A number of investigators (6, 29, 31) showed that the enzymatic activity of VCN and IVN is present at pH 7.0.

**Measurement of Sialic Acid.** Warren (43) originally described the thioarbituric acid method of sialic acid assay from tissues. Subsequently, Aminoff (2) described a slightly modified method which was followed throughout. To each 1.0-ml aliquot of supernatant collected after enzymatic digestion of cells, 0.5 ml of periodate reagent was added and incubated for 30 min at 37° in a water bath. The excess periodate was then reduced with 0.4 ml of sodium arsenite. After the color of iodine had disappeared (30 sec to 1 min), 4 ml of thioarbituric acid reagent was added, mixed well in a covered test tube, and heated in a boiling water bath for 10 min. The colored solutions were then cooled in ice water and shaken with 2.5 ml of acid butanol. The separation of the 2 phases was facilitated by centrifugation at 800 X g for 5 min, and the colors in the butanol layer were compared at 549 nm in a Beckman spectrophotometer. Sigma Type IV N-acetylneuraminic acid dissolved in BSS was used as standard.

**RESULTS**

**Kinetics of the Release of Sialic Acid from Normal Cells by VCN or IVN.** Rabbit lymph node cells (2 X 10^8) were incubated with increasing concentrations of VCN or IVN at 37° for 1 hr. After incubation, the cells were centrifuged and the supernatants were assayed for sialic acid. Chart 1 represents the results of the mean of 5 separate experiments. Concentrations greater than 25 units of VCN per 2 X 10^8 cells did not release greater amounts of sialic acid from cell surfaces. Similarly, raising the concentration of IVN did not increase the amount of sialic acid released. In both of these cases, 25 or 50 units of enzyme released almost the same amount of sialic acid as was released by 100 units. Prolongation of the incubation time up to 6 hr did not cause the release of greater amounts of sialic acid into the medium. Therefore, in all of the subsequent experiments, 25 units of enzyme per ml were used to treat the various cell types. A 1-hr incubation with low concentrations of VCN appears to represent an estimate of the number of easily available, surface sialic acid residues. Previous studies showed that such treatment alters the electrophoretic mobility (1, 24, 25, 41, 42, 44) and other surface characteristics of cells (4, 6, 9–13, 17, 19, 26–31, 35–40). Therefore, it is likely that easily removable sialic acid residues are those most closely associated with the cell membrane.

**Comparative Release of Sialic Acid from Normal Cells by VCN and IVN.** Normal mouse nucleated and nonnucleated cells (2 X 10^8) were incubated with 25 units of VCN and IVN per ml in a total volume of 2 ml at 37° for 1 hr. The supernatants were assayed for sialic acid, and the results are shown in Table 1. Sialic acid was released in much greater quantity during VCN treatment than during IVN treatment. The ratio of VCN-susceptible to IVN-susceptible residues ranged from 3:1 to 7:1 in various normal cells.

VCN hydrolyzes 2—3, 2—6, and 2—8 linkages between the sialic acid and mucopolysaccharides, while IVN hydrolyzes 2—3 and 2—8 linkages (14, 15). It appears that the 2—6 glycosidic linkage is the predominant linkage by which sialic acid is bound to normal cells. The 2—3 and 2—8 linkages appear to be relatively uncommon on the cell surface, as evidenced by the relatively small amount of sialic acid released by IVN. Since, by definition, an IVN unit will release more sialic acid from a standardized substrate than will a VCN unit, and since an increase in the concentration of IVN did not lead to further increases in sialic acid release, it would appear that the accessible sialic acid residues are bound primarily by the 2—6 linkage.

**Comparative Release of Sialic Acid from Mouse Tumors by VCN and IVN.** Single-cell suspensions (2 X 10^8) of various mouse tumors were incubated with 25 units of VCN and IVN per ml in a total volume of 2 ml at 37° for 1 hr. The supernatants were assayed for sialic acid, and the results are presented in Table 1. There appear to be more VCN- and IVN-susceptible sialic acid residues on malignant cells than on various normal cells. However, it cannot be precisely stated (for want of suitable controls) whether malignant cells always possess larger quantities of enzyme susceptible cell surface sialic acid than their normal counterparts or whether this has any correlation with malignancy.

**DISCUSSION**

The results clearly demonstrate that cells possess a larger proportion of VCN- than IVN-susceptible sialic acid residues. The VCN-susceptible sialic acid in various mouse cells is about 3-fold more than the available IVN-releasable sialic acid on the cell surface (the only exception being thymocytes, in which the ratio is 7:1).

It is probable that the sialic acid molecules readily released from cell surfaces by enzyme digestion are those associated with the cell membrane, since surface properties of cells (1, 4, 6, 9–13, 17, 19) are altered by incubation with this enzyme. Cell viability, on the other hand, is not altered by incubation with VCN or IVN (26–30, 32, 46). Whether the enzyme-susceptible sialic acid residues are solely those associated with the membrane cannot be precisely determined. It is possible, however, that the enzyme enters the cell and sialic acid is released from it under certain circumstances. It is equally possible that the metabolically active cell repairs its cell
malignant cells (4-7, 10-13, 19, 35-40) will increase their orosomucoid (25, 45) and from both normal (19, 35, 38) and determinants. It is known that sialoglycoproteins are very poor acid residues on the surface of malignant cells is subject to sialic acid on their surface, and the 2—6glycosidic linkage immunogenicity. However, it is not known whether an immunogens (22, 33, 34). Removal of sialic acid residues from predominates as it does in normal cells.

The biological role of the relative increase in terminal sialic acid residues on the surface of malignant cells is subject to speculation. Apffel and Peters (3) suggested that 1 biological role of sialoglycoproteins may be the repression of antigenic determinants. It is known that sialoglycoproteins are very poor immunogens (22, 33, 34). Removal of sialic acid residues from orosomucoid (25, 45) and from both normal (19, 35, 38) and malignant cells (4—7, 10—13, 19, 35—40) will increase their immunogenicity. However, it is not known whether an increase in the sialic acid content on malignant cell surfaces has any correlation with cancer.

REFERENCES

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