Effect of Enzymatic Removal of Cell Surface Sialic Acid on the Adherence of Walker 256 Tumor Cells to Mesothelial Membrane

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SUMMARY

When cells of the Walker 256 rat tumor were incubated in vitro with neuraminidase, free sialic acid accumulated in the medium without detectable loss of tumor cell viability, as indicated by the unchanged survival of rats given i.p. injections of 50, 100, or 250 cells. For determination of the effect of neuraminidase treatment of the tumor cells on their adherence to living mesothelium, 1 testis of each of a group of rats was bathed, under standard conditions in vitro, in a suspension of either untreated or neuraminidase-pretreated tumor cells, at each of several cell concentrations. The testis was washed and, with its circulation still intact, placed in the peritoneal cavity of the same animal, and the incision was closed. In rats exposed to 100 to 4,000 cells/ml, host survival was longer when the cells had been pretreated with neuraminidase. Similarly, after the direct i.p. injection of 500 to 10,000 cells, host survival was longer after neuraminidase treatment of the tumor cells. The effect was shown to be due to reduced attachment of the treated tumor cells and did not involve any increase in tumor cell antigenicity. The results indicate that the adherence of dissociated Walker 256 tumor cells to mesothelial membrane depends in part on the presence of sialic acid at the tumor cell surface.

INTRODUCTION

Circulating tumor cells disappear rapidly from peripheral blood (20). Before they show any evidence of proliferation or invasion of the host tissues, the cells come to rest and attach to the vascular endothelium of capillaries and arterioles (28). Observations in rabbit ear chambers have shown that the attachment of tumor cells to capillary endothelium can be independent of both the capillary diameter and the rate of blood flow (27), suggesting that embolic tumor cells are not always arrested by simple mechanical filtering. The adherence of circulating tumor cells to a substrate may therefore be an important stage of metastasis formation. Clinically, the adherence of tumor cells to peritoneum is also well known in patients with disseminating gastric or ovarian carcinoma (2). However, little is known about the mechanism of initial adherence of tumor cells to peritoneum because in other studies the detection of their adherence depended on the morphological recognition of attached cells at a relatively late stage of implantation (4).

A superficial coating of acidic glycoprotein has been demonstrated histochemically on TA3 mouse ascites tumor cells (10), transformed hamster embryonic cells (9), and normal rat cells (16, 17), and an analysis of the products of proteolytic digestion of Ehrlich ascites tumor cells has shown that surface glycoprotein is associated with their cell membrane (6). There is some evidence that surface glycoprotein may be involved in the adherence of tumor cells to vascular endothelium in tumor metastasis. A study on the formation of metastases from TA3 tumor cells pointed to the importance of a Hale-positive (acidic carbohydrate) coating on both the tumor cells and the endothelium of mouse lung capillaries (10), and reported that this coating could be removed by incubation with NMase.2 The authors found that the number of metastases developing from TA3 ascites cells, injected i.v., was decreased by the prior i.v. injection of NMase. This antimetastatic effect of NMase was attributed to an effect on the adherence of the injected tumor cells to the vascular endothelium. However, it was later reinterpreted by the same authors as being due to a reduction in platelet count (11).

Some information on tumor cell adherence has been obtained from cell culture systems. Measurements of the effect of NMase showed that sialic acid contributes to the adherence of cells to a glass substrate (26). This property of sialic acid may be related to its function as a primary cell surface charge determinant, for NMase also reduces electrophoretic mobility (23). The reduction in surface charge obtained with this enzyme is due to the removal of the charged carboxyl groups of sialic acid from the cell surface (23). NMase also has an effect on the invasiveness of polyoma-transformed fibroblasts in organ culture (29). Since there might thus be a basis for postulating that surface sialic acid could confer adhesive

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2 The abbreviations used are: NMase, neuraminidase; NANA, N-acetylneuraminic acid.
properties on tumor cells, we decided to test this hypothesis.

The Walker 256 rat carcinosarcoma is a tumor which is well known for its pronounced tendency to metastasize (1, 5). Its cells are easily dispersed by mechanical means, making the use of enzymes for dissociation unnecessary, so that any artificial change of the tumor cell surface due to dispersal may be considered minimal. Our preliminary work with histochemical stains revealed an irregular layer of acidic glycoprotein on the dispersed cells, the staining of which was reduced by incubation with NMase, suggesting that it contained enzyme-labile sialic acid.

To study the function of the surface glycoprotein in tumor cell adhesiveness, we devised an experimental model. The tunica vaginalis of the rat testis is a squamous mesothelial membrane, continuous with peritoneum, that can be manipulated outside of the body and exposed to tumor cell suspensions without trauma or interference with blood supply (A. C. Wallace, personal communication). It is therefore suitable for use as a substrate to test tumor cell adherence. After in vitro exposure of the testis to a counted tumor cell suspension, the testis can be washed free of unattached cells and placed in the peritoneal cavity of the same animal. The number of animals that die with ascites tumors and the times until death can then be used as indices of tumor cell adherence to the testicular mesothelium. In this experimental model, tumor cell adherence to a viable membrane takes place in vitro under standardized conditions, changes in the attachment of small numbers of cells can be detected, and the effects of agents on adherence can be studied over a short time scale and in the absence of any systemic effects. The present paper reports the results of experiments designed to determine the effect of NMase on the adhesiveness of Walker tumor cells, with the use of this model system.

MATERIALS AND METHODS

Rats. Adult male Sprague-Dawley albino rats, 150 to 250 g, were obtained from Sprague-Dawley, Inc., Madison, Wis.

Tumor. Walker 256 carcinosarcoma, serially transplanted i.m. in Sprague-Dawley rats, was used for this study. Minced, nonnecrotic tumor was shaken with Medium 1066 (Difco Laboratories, Detroit, Mich.), containing 6% horse serum, and strained through gauze. Cells were diluted with 0.01% trypan blue (Edward Gurr, London, England) in phosphate-buffered 0.9% NaCl solution, pH 7.1, for counting in a hemocytometer. Only tumor cells that excluded the dye and showed no morphological signs of damage were counted.

Irradiation. A dual 60Co source was used to irradiate rats and tumor cells. Rats received 450 rads whole-body γ-radiation 1 day prior to tumor cell injection. Tumor cell suspensions received 15,000 rads immediately before injection.

Exposure of Testicular Mesothelium to Tumor. Each rat was anesthetized with Nembutal and 1 testis was taken out through a scrotal incision and placed, with its circulation intact, in a small dish of NaCl solution. The mesentery attaching the cauda epididymis to the testis was cut and a thin plastic circular diaphragm, with a slit running radially from its outside edge to a central hole 0.5 cm in diameter, was placed around the testicular-epididymal junction. This held the epididymis out of the way during the subsequent exposure of the testis to suspended tumor cells. The spermatic cord was left intact to maintain the blood supply to the testis throughout the procedure. The rat was placed horizontally over a chamber in which the testis could be bathed, under sterile conditions, in a stirred suspension of tumor cells at constant temperature (35°C). The apparatus is illustrated in Chart 1. The testis was immersed for 5 min in the counted suspension of tumor cells, in Medium 1066 with 6% horse serum, and washed twice with 0.9% NaCl solution to remove all the unattached tumor cells. The rat was then removed from the platform. The original incision was extended into and along the inguinal canal as far as the deep inguinal ring. Following insertion of the lower blade of the scissors into the peritoneal cavity through the deep inguinal ring, a 1-cm cut was made in a cephalic direction through the ventral body wall. To permit insertion of the exposed testis into the peritoneal cavity, a hemostat was clamped to the attached epididymal adipose tissue. The hind limbs were elevated to permit relaxation of the ventral abdominal wall musculature and widening of the incision, facilitating the entry of the testis into the peritoneal cavity. The hemostat was inserted through the incision and the testis was drawn gently into the cavity. This procedure obviated any direct handling of the testicular mesothelium at any time during its exposure to tumor cells. Finally, the abdomen was closed with skin clips, taking care to avoid any obstruction of the urethra.

Chart 1. Apparatus for the exposure of testicular mesothelium to tumor cell suspensions. The testis was exposed on Side A to NMase-treated cells and on Side B to untreated cells. d, plastic diaphragm to retain epididymis; h, heating tape; m, stirring magnet; ms, magnetic stirrer; t, exposed testis.
Enzyme Treatment. NMase, isolated from *Vibrio cholerae* filtrate, was obtained from Behringwerke, Germany. This purified preparation is a glycosidase which catalyzes hydrolytic cleavage of the 2,3- and 2,6-O-glycoside bonds between the C-2 of terminal NANA and the C-3 or C-6 of the underlying amino sugar residue (8), and is specified free of all proteolytic, aldolase, and lecithinase C activity. One unit of its enzymatic activity is defined as the amount of enzyme that releases 1 μg NANA from an α1-glycoprotein substrate in 15 min at 37°. For treatment with NMase, approximately 5 × 10⁸ suspended tumor cells were shaken for 1 hr at 37° with 50 units NMase/ml Medium 1066 containing 6% horse serum, pH 7. Sialic acid released to the medium was estimated by the thiobarbituric acid method (24). A similar concentration of control tumor cells was shaken for 1 hr at 37° in the same medium (pH 7), but containing 20% enzyme buffer (0.05 M sodium acetate-acetic acid-buffered 0.9% NaCl solution, pH 5.5, with 1 mg/ml calcium chloride) instead of enzyme. The required cell concentrations were prepared by appropriate dilution of these standard suspensions in Medium 1066 with 6% serum.

Histochemical Staining. Smears (fixed with formol sublimate) and sections were stained for acidic carbohydrate with 1% Alcian blue in 0.01 N HCl, pH 2.2 (30), by the Hale colloidal iron procedure (14) and with N,N-dimethyl-p-phenylenediamine (21).

Expression of Results. The survival of rats with Walker tumor cells proliferating intraperitoneally may be expressed as a curve of the log percentage survival plotted against time after the tumor cells are given (see Chart 2). This curve is composed of a horizontal portion representing 100% survival of the animals, followed by a sloping linear portion which expresses their exponential mortality with tumor (P. Richardson, unpublished data). The curve can therefore be described in terms of 2 parameters: (i) the minimum latent interval, which extends from the time when tumor cells are given to the animals until the 1st death with tumor (strictly, the point at which a line extrapolated from the sloping portion of the curve meets the 100% survival line) and (ii) the slope of the exponential portion of the curve (b, the regression coefficient of log percentage survival on time).

It was found that the minimum latent interval varied only slightly with variations of tumor cell dose, while the slope, b, was very sensitive to any changes in the number of tumor cells given to the animals. For this reason, the value of the coefficient b was used throughout this paper as an index of mortality with tumor. Comparisons between experimental groups have been made on the basis of the respective values of the coefficient b, using Student's t test to determine probabilities.

RESULTS

Effect of NMase on Adherence to Testicular Mesothelium. An experimental series was designed to test directly whether the enzymatic removal of surface sialic acid from tumor cells changed the number of cells adhering to a normal membrane. Preliminary work showed that rat testes, with their circulation intact, could be exposed *in vitro* outside the body for a period of 5 min and placed in the peritoneal cavity without detectable trauma to the mesothelium, provided that the mesothelium was immersed in 0.9% NaCl solution and not abraded during the exposure. One testis of each of a group of rats was then exposed to a tumor cell suspension, washed thoroughly, and placed in the peritoneal cavity of the same animal. When examined 3 days later, the adherent tumor cells were seen to have proliferated and spread over the testis as a monolayer. In many areas, tumor cells had displaced the mesothelial cells, causing disruption of the mesothelial membrane. The tumor cells subsequently invaded the epididymal adipose tissue, pancreas, and abdominal wall, and formed extensive hemorrhagic ascites tumors. The rats died in approximately 3 weeks. We therefore concluded that the dissociated tumor cells adhering to normal testicular mesothelium *in vitro* could proliferate *in vivo* and cause death of the hosts.

We were thus in a position to determine the effect of treating the tumor cells with NMase on the mortality with tumor following the exposure of a testis to dissociated tumor cells. One testis of each of 2 groups of 16 to 20 rats was exposed for 5 min *in vitro* to either untreated tumor cells or tumor cells incubated for 1 hr with NMase (50 units/ml). After washing, the exposed testis was placed in the peritoneal cavity of the same animal and the subsequent survivals of the untreated and NMase-treated tumor cell groups were compared. Graded concentrations of tumor cells, *i.e.*, 100, 250, 2000, 3000, 4000,
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Effect of NMase treatment of Walker tumor cells on survival of hosts following exposure of testicular mesothelium to different cell concentrations

For each cell concentration, after a period corresponding to the minimum latent period, log percentage survival (Y) decreased linearly with time (X), fitting the regression equation $Y = a + b X$ with a correlation coefficient ($r$) $\geq 0.90$. Data are given for independent experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of tumor cells/ml to which testis was exposed</th>
<th>Untreated tumor cells</th>
<th>NMase-treated tumor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mortality rate ($b \times 10^{-3}$) 95% confidence limits of $b$ (X $10^{-3}$)</td>
<td>Mortality rate ($b \times 10^{-3}$) 95% confidence limits of $b$ (X $10^{-3}$)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>30.6 17.5-46.6 2.8* 0.9-6.5</td>
<td>30.6 17.5-46.6 2.8* 0.9-6.5</td>
</tr>
<tr>
<td>2</td>
<td>250</td>
<td>30.4 18.3-42.5 5.2* 0.6-9.8</td>
<td>30.4 18.3-42.5 5.2* 0.6-9.8</td>
</tr>
<tr>
<td>3</td>
<td>3000</td>
<td>19.6 15.0-24.3 7.1 5.1-9.1</td>
<td>19.6 15.0-24.3 7.1 5.1-9.1</td>
</tr>
<tr>
<td>4</td>
<td>3000</td>
<td>39.1 28.3-50.0 19.1 11.2-26.9</td>
<td>39.1 28.3-50.0 19.1 11.2-26.9</td>
</tr>
<tr>
<td>5</td>
<td>4000</td>
<td>54.9 43.5-66.4 22.4 18.4-26.3</td>
<td>54.9 43.5-66.4 22.4 18.4-26.3</td>
</tr>
<tr>
<td>6</td>
<td>5000</td>
<td>50.3 37.4-63.2 34.2 28.0-40.5</td>
<td>50.3 37.4-63.2 34.2 28.0-40.5</td>
</tr>
<tr>
<td>7</td>
<td>7000</td>
<td>37.3 17.5-57.1 24.2 10.6-37.7</td>
<td>37.3 17.5-57.1 24.2 10.6-37.7</td>
</tr>
</tbody>
</table>

*0.01 $> p > 0.001$.  
*p $< 0.001$.  
*0.05 $> p > 0.01$.  

5000, and 7000 cells/ml, were applied. Although animals were routinely exposed alternately to either control or treated cells at each cell concentration, it was not possible to expose tests of control and treated groups to more than 1 cell concentration in any 1 day.

Table 1 shows the effect of NMase treatment of the tumor cells on host survival. When suspensions of 100, 250, 2000, 3000, or 4000 cells/ml were applied, survival was greater in the groups exposed to NMase-treated cells. This effect was not seen at higher cell concentrations (5000 and 7000 cells/ml).

Effect of NMase on Intraperitoneal Proliferation of Tumor Cells. The exposure of testicular mesothelium to tumor cell suspensions was followed by 2 separate processes. During an initial attachment phase, the tumor cells adhered to the mesothelium in vitro. Then, when the exposed testis was placed in the peritoneal cavity of the host, the adherent tumor cells proliferated i.p. to form ascites tumors. We decided to study the proliferative stage separately, by injecting the tumor cells directly into the peritoneum. An experimental series was planned to test whether pretreatment with NMase had any effect on the survival of rats given i.p. injections of the cells. One group of 18 to 24 rats was given i.p. injections of untreated cells, while the other was given i.p. injections of the same number of cells after 1-hr incubation with NMase (50 units/ml). Graded cell doses, i.e., 50, 100, 250, 500, 1,500, and 10,000 cells, were injected into groups of the same size. The subsequent survivals of the untreated and NMase-treated tumor cell groups are compared in Table 2. With 50, 100, or 250 cells i.p., the mortality rate with tumor was similar in both groups. With 500, 1,500, and 10,000 cells i.p., however, survival was greater in the groups receiving the NMase-treated cells.

The results show that after either the application of 100 to 4,000 tumor cells/ml to testicular mesothelium or the i.p. injection of 500 to 10,000 cells, host survival was longer when the cells had been pretreated with NMase. This finding could be interpreted in several ways. First, it could indicate that NMase acted by reducing the adherence of the tumor cells to mesothelial membrane. Second, the NMase treatment could have reduced the viability of the tumor cells. A 3rd possibility was that the enzyme treatment had increased the antigenicity of the tumor cells, resulting in an increased immunological destruction of the treated cells by their hosts. Such an effect on antigenicity has been reported with the TA3 (19) and Landschütz (7, 8) ascites tumors.

Effect of NMase on Viability of Tumor Cells. When the survivals of rats given i.p. injections of small numbers of NMase-treated cells (50, 100, and 250 cells) were compared with those of rats receiving the same number of the untreated cells, there were no differences in survival between the treated and control groups (see Experiments 1 to 3 in Table 2). Any impairment of proliferative capacity due to NMase treatment would have become apparent after equal small numbers of treated and control tumor cells had proliferated in vivo, and should have been detectable by the increased survival of the group receiving the treated cells. Since assay of the incubation medium showed that sialic acid was released from the tumor cells under the standard conditions of incubation (Chart 3), it is concluded that the removal of enzyme-labile sialic acid from Walker tumor cells did not affect their viability, as measured by their capacity to proliferate in vivo.

NMase-treated tumor cell suspensions were checked for gross damage by the addition of 0.01% lissamine green, and also for NMase uptake by using the fluorescein-labeled enzyme (15). The proportion of cells exclud-

Table 2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of tumor cells injected i.p.</th>
<th>Mortality rate ($b \times 10^{-3}$) 95% confidence limits of $b$ (X $10^{-3}$)</th>
<th>Mortality rate ($b \times 10^{-3}$) 95% confidence limits of $b$ (X $10^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>3.8 0.0(4)- 6.7 3.8</td>
<td>0.0(4)- 6.7 3.8</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>11.6 8.0-15.3 10.0</td>
<td>5.4 -14.6</td>
</tr>
<tr>
<td>3</td>
<td>250</td>
<td>32.3 29.4-35.2 32.3</td>
<td>29.4 -35.2</td>
</tr>
<tr>
<td>4</td>
<td>500</td>
<td>50.0 42.0-58.0 14.2</td>
<td>9.2 -19.2</td>
</tr>
<tr>
<td>5</td>
<td>1,500</td>
<td>41.9 38.6-45.1 24.2</td>
<td>22.4 -26.0</td>
</tr>
<tr>
<td>6</td>
<td>10,000</td>
<td>108.9 85.5-132.3 39.6</td>
<td>31.3 -48.0</td>
</tr>
</tbody>
</table>

*p $< 0.001$.  

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ing the dye was consistently equal to or slightly higher than that in untreated control suspensions. Fluorescein-labeled NMase was not demonstrably taken up by the tumor cells, even when applied for 5 hr at 5 times the enzyme concentration routinely used. Normal rat neutrophils provided a positive control, since fluorescence microscopy showed that when exposed to this concentration of labeled enzyme (250 units/ml), about 1 in 5 neutrophils took it up in the form of very small cytoplasmic droplets (G. Casson, unpublished data). These findings suggest that the NMase-treatment used could release sialic acid from Walker tumor cells without entering the cells or causing any gross damage.

**Effect of NMase on Antigenicity of Tumor Cells.**

There was also the possibility that the increased survival, obtained with NMase-treated tumor cells when they were applied to the testis (100 to 4,000 cells/ml) or injected i.p. (500 to 10,000 cells), might have an immunological basis. If the enzyme treatment were to render this tumor more antigenic in its host (with which it is not syngeneic), the ensuing increase in the immune response of the host might delay growth of the tumor, thereby prolonging the survival of the hosts. An experiment was therefore designed to determine whether the decreased mortality rate obtained with the treated cells was due to an increase of their antigenicity. Groups of 30 rats were injected i.p. with 10⁶ lethally irradiated tumor cells (18), which were either untreated or pretreated with NMase, mixed with 10⁴ viable untreated cells. Chart 4 shows that the addition of 10⁶ untreated cells significantly reduced the mortality rate while the addition of 10⁶ NMase-treated cells had no effect on mortality rate.

To test whether this was an immunological effect, the experiment was repeated with hosts that were given sublethal total-body radiation (450 rads) 1 day prior to injection. The result is shown in Chart 5. In the irradiated...
hosts, the mortality rate was the same for all groups. Since the prolongation of survival through the addition of $10^8$ untreated cells was eliminated by the total-body radiation, it is concluded that the effect of these added cells on survival was essentially an immunological one. Because the addition of NMase-treated cells had no effect on mortality rate, while the same number of untreated cells reduced it, the NMase-treated cells must have been less antigenic than the untreated cells. Therefore, the reduction in mortality rate observed with NMase-treated cells in the previous experiments could not have been due to increased antigenicity of the enzyme-treated cells.

**DISCUSSION**

Walker tumor cells and rat testicular mesothelium stained positively for surface acidic glycoprotein. The acidic staining property of the surface coating is largely attributable to the highly acidic free carboxyl groups of sialic acid (pKₐ 2.6), which are known to confer a net negative charge on the cell surface (23). In the present study, relatively mild NMase treatment of Walker tumor cells removed sialic acid (approximately $10^8$ molecules NANA from each cell) without concomitant reduction of proliferative capacity of the cells in vivo, as measured by the ability of small cell doses (50, 100, or 250 cells) to kill i.p.-injected hosts. The unchanged mortality from small numbers of cells injected i.p. and the lissamine green exclusion tests have provided evidence that the NMase treatment used did not damage the tumor cells. Since there was no demonstrable uptake of the fluorescein-labeled enzyme, it is likely that, at the concentration used in this study (50 units/ml), NMase was excluded and therefore only able to act on the cell surface. It follows that the enzyme-labile sialic acid was probably either in the surface glycoprotein coating or the cell membrane. NMase-labile sialic acid has been demonstrated at the surface of liver cells by electron microscopy after colloidal iron staining (3).

An important factor to be considered is the resynthesis of surface sialic acid on the tumor cells following their treatment with NMase. On HeLa cells, the receptor sites for Newcastle disease virus are resynthesized after NMase treatment with a half-time for recovery of 5.9 hr (13). Since NANA contributes to NMase-sensitive virus receptor sites (25), this short recovery time may indicate that surface NANA is resynthesized fairly rapidly. To minimize the possible effect of resynthesis of enzymatically removed NANA, we restricted the period of exposure of testicular mesothelium to suspended tumor cells to 5 min. The treated tumor cells (as well as the untreated control cells) were applied to the testis no later than 2 hr after enzyme treatment, and usually within 1 hr. The short time scale used should have obviated any significant resynthesis during the test period.

The increased survival of rats obtained when testicular mesothelium was exposed to NMase-treated tumor cells suggested a reduced adherence of the treated cells to mesothelial membrane. Although dilution did not entirely eliminate the NMase in the treated cell suspensions, its concentration was always reduced by a factor of 1,000 or more, giving a maximal level of 0.05 unit/ml. Any possible effect of brief exposure of the mesothelial membrane to such a low concentration of the enzyme may be considered of secondary importance in affecting tumor cell adherence. The adhesiveness-reducing action of NMase on tumor cells could also have accounted for the increased survival observed when 500 to 10,000 treated cells were injected directly i.p. However, no difference in survival was found between the treated and control cell groups with an i.p. dose of less than 500 cells. These apparently contradictory data can be fitted to a model of ascites tumor formation if it is assumed that the adherence of the injected tumor cells to host tissues gives them a proliferative advantage over nonattached tumor cells. When small numbers of cells (up to 500) were injected i.p., the frequency with which they would have been able to make random contact with peritoneum would have been relatively low. Since the time necessary for all the cells to encounter a site for adhesion could thus have been large compared with the time of resynthesis of surface sialic acid, many of the tumor cells may have adhered after the resynthesis had taken place. At this time, there would no longer be any reduction in surface sialic acid, and no difference in survival between the treated and untreated cell groups would have been expected. On the other hand, when larger numbers of cells were injected i.p., adherence could have occurred more rapidly relative to the time of resynthesis, as a result of the greater probability of random contact of tumor cells with peritoneum. Under these conditions, it would be expected that any difference in the ability of the treated tumor cells to adhere would have become apparent as a difference in host survival, and this was shown to be the case. The results indicate that, with i.p. doses of 500 cells or more, some tumor cell adherence occurred before the enzyme-labile surface sialic acid could be resynthesized on the treated cells (Table 2).

The mortality rate with tumor following the exposure of 1 testis to untreated tumor cells was found to remain relatively constant when different cell concentrations were applied (Table 1). The overall mortality rate corresponded approximately to that resulting from 250 to 1500 untreated cells injected directly i.p. (Table 2), suggesting that between 200 and 1500 untreated cells adhered to each testis at each of the cell concentrations applied (100 to 7000 cells/ml).

Because its cells could be readily dispersed with a minimum of trauma, the Walker 256 rat tumor seemed to be most suitable for this study. This tumor, however, was not syngeneic with its host, and, as expected, there was a considerable immunological response to the tumor by its host. The present study showed that the enzymatic removal of NANA from Walker tumor cells rendered them less antigenic, for NMase-treated tumor cells were less effective than untreated tumor cells in reducing the...
rate of mortality from the tumor. It has been shown that such a reduction in mortality rate by injecting admixed lethally irradiated cells is due to active immunization of the host against the foreign antigens carried by the tumor cells (18). A somewhat similar effect of NMase was also observed on erythrocytes, in which the enzyme decreased the antigenicity of the glycopeptide M and N antigens (12, 22). In this respect, cells of the Walker 256 tumor appear to show an important difference from TA3 (19) or Landschütz (7, 8) ascites cells. Studies with these tumors have suggested that the effect of NMase on these cells was to reveal potential surface antigens that were ordinarily undetected by the host because they were masked by a thick glycoprotein coat. This exposure of antigen would lead to an increase in the destruction of the tumor cells by the immune response of the host, thereby prolonging host survival. Such an increase in antigenicity would result only if the coat, complete and uniform on the untreated cell, became perforated by channels large enough for hidden underlying cell surface antigens to be revealed. Since the coating on Walker tumor cells is irregular and incomplete, perhaps due to its uneven distribution during the dissociation of the individual tumor cells, such a process of antigen revelation would not be a likely consequence of NMase treatment. An increase of antigenicity would therefore not be expected.

An effect of NMase on tumor cell antigenicity or viability can therefore be ruled out as explanations for the reduced mortality from Walker 256 ascites tumors produced by the i.p. injection of 500 to 10,000 NMase-treated cells, or the application of 100 to 4,000 NMase-treated cells/ml to testicular mesothelium. The present study thus indicates that the action of NMase was to reduce the adhesiveness of the treated tumor cells.

From the present work, 2 functions can be ascribed to the surface sialic acid on Walker tumor cells. First, its presence contributes to the antigenicity of the cells, for they become less antigenic when it is removed. Second, it is implicated in the adherence of dissociated tumor cells to membranes. We have evidence that it is involved in the implantation of tumor cells on peritoneum during the formation of ascites tumors and suggest that it might be similarly involved in metastasis formation following the dissemination of cells from primary malignant tumors. The possible relationship between the antigenicity and adhesiveness of tumor cells is a problem that may merit further investigation.

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