ANTIMETASTATIC EFFECTS ASSOCIATED WITH PLATELET REDUCTION*

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We have previously reported that the number of metastases resulting from the inoculation of TA$_3$ ascites tumor cells into mice could be reduced by 50 per cent if the mice were first injected with neuraminidase.[1] It was assumed that this effect probably reflected the action of neuraminidase on the sialic acid coat of tumor or host endothelial cells, since it was believed that mutual stickiness between these cell types was important in metastasis formation.[1]

Since the uncoating of tumor cells by neuraminidase prior to injection into the host seemed to have very little antimetastatic effect,[1] attention was directed to the effects of neuraminidase on the host. The present paper reports the unexpected finding that neuraminidase *in vivo* produces thrombocytopenia, with a close correspondence between the decrease in platelet levels and the decrease in metastases. Further experiments, in which the concentration of circulating platelets was varied by other agents, confirm the association between platelet levels and metastases, and suggest that the previously observed antimetastatic effect of neuraminidase may be at least partially mediated by its effect on the platelets.

Materials and Methods.—TA$_3$ ascites tumor cells[2] were grown in the peritoneal cavity of CAF$_1$/Jax female mice, each receiving 2.0–2.5 × 10^5 or 3.0–3.5 × 10^5 tumor cells suspended in 0.2 ml of medium. They were killed 14–16 days later, and ether and lung metastases were counted.

Neuraminidase was given i.v. at different intervals before tumor inoculation; each mouse received a single dose of 0.2 ml of a commercial solution of neuraminidase from *Vibrio cholera* (Behringwerke) containing 500 units per ml. Each unit was capable of releasing from α^1-glycoprotein 1 μg of sialic acid as N-acetylneuraminic acid at 37°C in 15 min at pH 5.5. It is reported to be free of proteinases, aldolase, and lecithinase C. Controls were injected with the diluent of the commercial neuraminidase.

When it became apparent that neuraminidase was preventing normal clot retraction and prolonging the bleeding time,[3] blood platelets were counted with a modified method of Nakeff and Ingram.[4] Briefly, we proceeded as follows. Peripheral blood was obtained from the tail vein of mice in 10-μl double oxalated capillary tubes (Scientific Products) and diluted to 20 μl with a phosphate-buffered saline (PBS) containing 0.1% ethylenediaminetetraacetate (EDTA). This new suspension was taken up into a 75-μl heparinized standard microhematocrit capillary tube (Scientific Products) followed by approximately 10 μl of Dow Corning silicone fluid no. 550 (specific gravity, 1.07000). This tube was sealed with Critoseal (Scientific Products), and a small gap of air was left between the seal and the silicone fluid. The tubes were centrifuged in an IEC hematocrit centrifuge for 30 sec. Then each tube was broken at the plasma-silicone interface, and the layer of platelets was suspended in 20 ml PBS with 0.1% EDTA and counted electronically with a Coulter counter.

In some experiments, platelets were transfused 24 hr after the treatment with neuraminidase and 4 hr before tumor inoculation. In this case, one group of neuraminidase-treated mice was injected i.v. with platelet-rich plasma (PRP) (about 1.2 × 10^8 platelets in 0.25 ml of plasma per mouse), and another similarly treated group with platelet-poor plasma

(PPP) (about 3×10^6 platelets in 0.25 ml of plasma per mouse). A third group, treated only with the diluent of neuraminidase, was transfused with the same concentration of PPP.

PRP for transfusion was prepared aseptically at room temperature with blood from 3-month-old CAF_1/Jax male mice. Ether-anesthetized mice were bled visually from the right heart with a disposable plastic syringe containing 0.2 ml of ACD acid solution as anticoagulant.[5] Between 1.0 and 1.6 ml of blood was obtained from each mouse. The blood from 20 donors was distributed among four polystyrene tubes (17×100 mm) and centrifuged at 1000 rpm ($225 \times g$) for 10 min in an International Centrifuge, model PR-2, at 25°C. The supernatant PRP was removed from each tube, pooled, and centrifuged again at 1000 rpm for 10 min to eliminate remaining erythrocytes and leukocytes. The residual blood from the first centrifugation was spun down at 2500 rpm ($1400 \times g$) for 15 min and the supernatant collected and used as PPP. The pH of both types of plasma was adjusted to 6.8 with 1 N NaOH. The electronic counts for the platelets ranged from 670×10^3 to 768×10^3/mm³ for PRP, and from 3.6×10^3 to 22×10^3/mm³ for PPP.

To prepare antiplatelet serum (APS), platelets from the PRP were washed and suspended in saline (10^9 per 5 ml of saline) and twice injected i.v. 14 days apart into adult New Zealand white rabbits weighing 4.5 kg. Seven days after the last injecion, rabbits were exsanguinated and serum was prepared. The serum was decomplemented at 56°C for 30 min and adsorbed for 1 hr at 37°C with an equal volume of packed CAF_1/Jax mouse red cells. Its activity against platelets was tested both *in vivo* and *in vitro*.

To investigate the APS effect upon metastases, CAF_1/Jax mice were injected i.p. with APS, each mouse receiving 0.05 ml of the serum. Control mice were injected i.p. with the same amount of normal rabbit serum, which was also decomplemented and adsorbed with red cells. Twenty-four hours later, mice were inoculated i.v. with 3.3×10^5 TA₃ tumor cells. One hour before tumor inoculation, platelets were counted electronically in ten uninoculated mice of each group. Tumor-inoculated mice were killed 15 days later and lung metastases were counted.

To study the activity of neuraminidase in circulation, mice were injected i.v. with a single dose of the enzyme (100 or 200 units) and bled at different intervals from the right heart. Because serum was difficult to obtain from the neuraminidase-thrombocytopenic mice, heparinized plasma (0.05–0.5 ml) was incubated at 37°C for 5 min in the presence of 0.2–0.4 ml of sialyllactose as substrate (1 mg/ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-maleate buffer at pH 5.5, containing 0.001 M $CaCl_2$ and 0.04% bovine serum albumin).[6] The free sialic acid was measured by the thiobarbituric method of Warren[7] after plasma proteins were precipitated with phosphotungstic acid.[8]

Results.—Neuraminidase reduced the number of lung metastases in every group treated with the enzyme, but the intensity of this effect varied according to the interval between treatment and tumor inoculation. The effect began as early as 1 hour after treatment, became increasingly stronger until it reached a maximum at 24 hours, and then steadily decreased. It affected both the percentage of mice with lung tumors and the mean number of metastases per group. The differences of the mean number of metastases for the 1-, 2-, 3-, 5-, 18-, and 24-hour-neuraminidase-treated groups as compared with the controls are highly significant (Table 1). The decreasing frequency of metastases with time of neuraminidase exposures up to an interval of 24 hours closely corresponds with a similar decline in neuraminidase activity in the blood as shown by the chemical assay of the enzyme activity in the plasma (Fig. 1).

The thrombocytopenic effect by neuraminidase was apparent 1 hour after treatment, increased with time, and reached its maximum 24 hours later. From 48 to 96 hours, the number of platelets increased steadily without, however,

TABLE 1. *Frequency of lung metastases and time of neuraminidase administration before
tumor inoculation.**

Groups	No. mice	Mice with metastases (%)	Mean no. metastases†	P
Control	162	92	5.85 ± 0.35	
Nase-1 hr‡	80	84	3.47 ± 0.46	<0.01
Nase-5 hr	30	67	2.46 ± 0.46	<0.01
Nase-24 hr§	106	57	1.37 ± 0.17	<0.01
Nase-48 hr	26	84	4.50 ± 0.77	>0.05
Nase-72 hr	30	76	3.86 ± 0.70	>0.05
Nase-96 hr	28	78	3.82 ± 0.73	>0.05

P = level of significance between the sample mean and the control mean.   Nase = neuraminidase.
* Mice were inoculated with 2.0–2.5×10^5 tumor cells.
† Mean ± standard error.
‡ Includes 20 mice Nase-treated 2 and 3 hr before tumor inoculation.
§ Includes 46 mice Nase-treated 18 hr before tumor inoculation.

attaining normal values.   The difference between the number of platelets in the
controls and neuraminidase-treated animals was statistically significant for any
interval of treatment (Table 2).

The effect of neuraminidase on thrombocytes at different intervals parallels the
effect of this enzyme on the number of metastases.   This is particularly striking
in the 24-hour-interval group, which shows a maximal drop both in platelets and
metastases (Fig. 2).

This antimetastatic effect of neuraminidase can be reversed by platelet trans-
fusion.   As shown in Table 3, neuraminidase decreased the number of metastases
in mice given platelet-poor plasma from 16.7 to 6.3 as expected.   If neuramini-
dase-treated mice were given platelet-rich plasma instead, the reduction in metas-
tases was much less, from 16.7 to 11.0.   That the transfusion of PRP is effective
in raising the number of platelets is shown by the results of another experiment,
where the mean number of platelets rose from 19×10^3 to 113×10^3/mm³ after
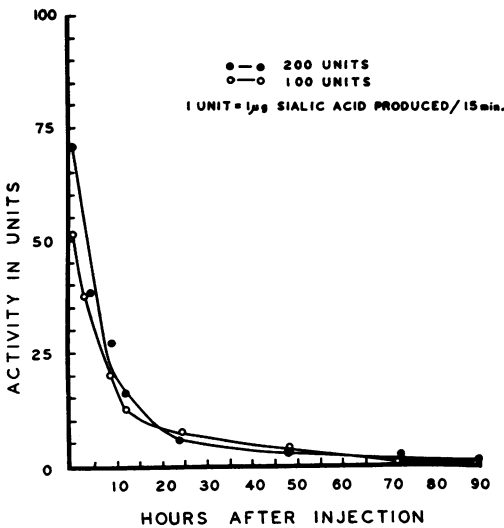the PRP transfusion (Table 4).   The transfusion at the platelet concentration

FIG. 1.—Plasma activity of neur-
aminidase. The neuraminidase ac-
tivity remaining in plasma is plotted
as a function of the time after injection
of either 200 or 100 units of the
enzyme.

TABLE 2.   *Effect of i.v.-administered neuraminidase on circulating platelets.*

Groups	No. mice	Mean no. of platelets ($\times 10^3$/mm³)*	P
Control	56	770 ± 32	
Nase-1 hr	15	413 ± 20	<0.01
Nase-3 hr	10	234 ± 16	<0.01
Nase-24 hr	45	48 ± 3	<0.01
Nase-48 hr	20	163 ± 14	<0.01
Nase-72 hr	15	302 ± 27	<0.01
Nase-96 hr	25	612 ± 32	<0.01

P = level of significance between the sample mean and the control mean.
Nase = neuraminidase.
* Mean ± standard error.

used did not return the number of circulating platelets to the normal control values (552 $\times$ 10³/mm³). However, the rise obtained is statistically significant.

Injection of antiplatelet serum produced a sharp drop in platelets, as expected.[9] Twenty-four hours after this injection, the platelets dropped from 570 $\times$ 10³ to 17 $\times$ 10³/mm³. Counts of metastases revealed a significant difference between APS-treated and control mice (Table 5).

Discussion.—On the assumption that adhesion of tumor cells to the vascular lining is a phenomenon determined by the sticky properties of their surface sialomucins,[10] we have investigated in the past the effects of neuraminidase on metastasis under two conditions. In one case, tumor cells were treated *in vitro* with neuraminidase, which effectively stripped sialic acid from their mucin coats; whereupon they were inoculated i.v. into untreated hosts. In the other case, neuraminidase was injected i.v. into the hosts before the inoculation of intact, coated tumor cells.
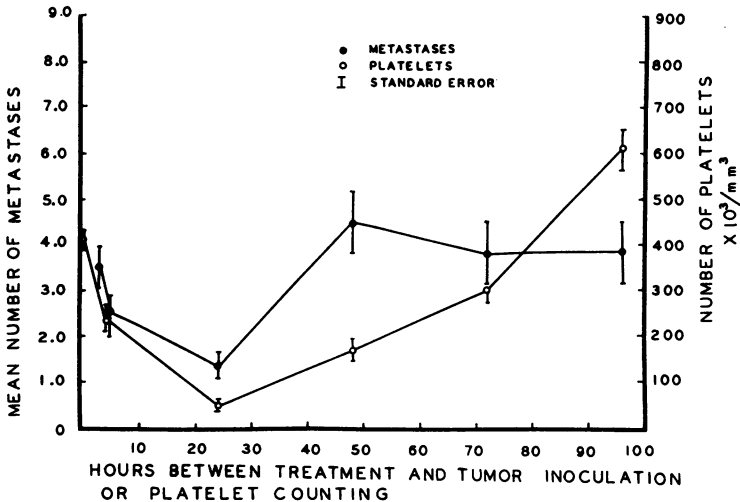

FIG. 2.—Effect of neuraminidase on circulating platelets and lung metastases. The number of lung metastases resulting from i.v. tumor cell inoculation is plotted as a function of the time after i.v. injection of 100 units of neuraminidase. Lung metastases were counted 14–16 days after tumor cell inoculation.

TABLE 3.   *Effect of platelet transfusion on metastasis in neuraminidase-treated mice.*

Group	Nase-treatment	Infused plasma	No. of mice	Mean no. of lung metastases[†]
1	None	PPP	21	16.7 ± 1.2
2	Yes	PPP	21	6.3 ± 0.7
3	Yes	PRP	24	11.0 ± 1.2

P difference between groups 1 and 2 < 0.01.   *P* difference between groups 2 and 3 < 0.01.
Nase = neuraminidase.
* Each mouse received 3.2×10^5 TA$_3$ tumor cells.
† Mean ± standard error.

We found that the enzyme reduced the number of metastases only when administered to the host before tumor inoculation.   Although this was an indication that the host cells might contribute more than the tumor cells to the observed reduction of metastases by neuraminidase, the participation of the latter was not ruled out because the possibility was entertained that the *in vitro* neuraminidase-treated tumor cells, once injected into the circulation, might regenerate their sialomucin coats and thus preserve their ability to initiate metastases.   Recoating of the tumor cells would not occur, however, if neuraminidase was already present in the circulation.   Since the enzyme was also effective in removing sialomucins from the vascular endothelium, it was originally thought that neuraminidase was antimetastatic by virtue of its capacity to affect both cell surfaces.[1]

Since the results with the *in vitro* neuraminidase-treated tumor cells might also indicate that changes of their surfaces by the enzyme is of little significance or of no significance at all for the antimetastatic effect, further studies were performed to investigate whether the host plays the dominant role in this phenomenon. For this purpose, intact, coated tumor cells were inoculated at different intervals after a single dose of the enzyme was administered by way of the tail vein.   It was assumed that its activity in the circulation would decrease with time, and that at a particular interval the host cells would be maximally affected, with little of the circulating enzyme available to interact with the injected tumor cells. This particular stage seems to be reached about 24 hours after neuraminidase administration, as indicated by direct assays of the enzyme in the plasma (Fig. 1).

The fact that the neuraminidase antimetastatic effect was most intense when there was less circulating enzyme at the time of tumor inoculation (as shown by Fig. 1, less enzymatic activity remains at 24 hours than at earlier intervals) is

TABLE 4.   *Effect of platelet transfusion upon number of platelets in neuraminidase-treated mice.*

Group	No. of mice	Nase-treatment	Infused plasma	Mean No. of Platelets $\times 10^3$/mm^3* Before transfusion[†]	After transfusion[‡]
1	12	None	PPP	564.4 ± 23.9	552.6 ± 37.3
2	20	Yes	PPP	25.7 ± 1.9	25.6 ± 1.7
3	22	Yes	PRP	19.4 ± 1.7	113.3 ± 4.8

P difference in group 3 before and after PRP transfusion <0.01. Nase = neuraminidase.
* Mean ± standard error.
† Platelets counted 24 hr after neuraminidase treatment; ten mice per group.
‡ Platelets counted 4 hr after plasma transfusion.

TABLE 5. *Effect of APS on number of lung metastases.*

Group	No. of mice	No. of mice with metastases	Mean no. of lung metastases*	P
Control	50	48	8.4 ± 0.5	
APS†	50	42	2.9 ± 0.3	<0.01

P = level of significance between the sample mean and the control mean.
* Mean ± standard error.
† APS administered 24 hr before inoculation of 3.3×10^5 TA$_3$ tumor cells.

strong evidence favoring the hypothesis that neuraminidase reduces the number of metastases by acting mainly through the host. A wholly unexpected finding added still further support to this conclusion. This was the discovery that neuraminidase *in vivo* produced a drop in blood platelets and that the intensity of this phenomenon at different intervals paralleled that of the antimetastatic effect (Fig. 2).

On further investigation of the role of platelets in metastasis, it was found that APS-induced thrombocytopenia also decreased the number of metastases (Table 5). In addition, transfusion with PRP reversed the antimetastatic effect by neuraminidase (Table 3). Results of these two experiments strongly indicate that platelets somehow are involved in the production of metastases. It remains to be investigated whether the decrease in platelets is wholly responsible for the drop in metastases, or whether additional factors are also necessary.

We do not know how platelets may participate in the formation of metastases in our particular system. Two well-known functions of platelets are involved in the processes of blood clotting and hemostasis.[11, 12] Since thrombosis or fibrin deposition around arrested tumor cells seems to be a necessary step in the formation of metastases, contributing either to the tumor cell attachment to the vascular endothelium or to their intravascular viability and growth,[13] it is reasonable to assume that one possible contribution of platelets to metastasis might be exercised through their normal role in coagulation. One of the platelet coagulating factors, platelet factor 4, which neutralizes heparin, might also influence metastasis, not only by preventing the heparin antithrombotic activity but also by maintaining a high lipemia.[13]

Another possible function of platelets in metastasis formation might be achieved through their physiological relationship to the vascular endothelium, which at present is not completely understood. It has been claimed by some authors[14, 15] that platelets contribute to surface or interendothelial-cementing substances, and there is evidence that these may be sulfated mucopolysaccharides.[16] As shown by very recent electron-microscopic studies, a reduction in platelets results in a widening of the interendothelial gaps.[17] If platelets in their entirety are utilized by the capillary bed, or if they are transferring sticky substances to the blood vessel wall, they might well contribute to metastasis by promoting vascular attachment of the circulating tumor cells.

Summary.—Results are presented which indicate that the number of metastases in mice can be decreased by reducing the number of host platelets before tumor inoculation. The result is similar whether the platelets are reduced by treatment with neuraminidase or with APS, and the effect can be reversed by

PRP transfusion. The mechanism of this effect is not known, but it is probably related to tumor cell attachment to the host vascular endothelium either by clot formation, promotion of surface stickiness, or both.

[1] Gasic, G., and T. Gasic, these Proceedings, **48**, 1172 (1962).

[2] Hauschka, T. S., *Trans. N. Y. Acad. Sci.*, **16**, 64 (1953).

[3] Firkin, B. G., G. Arimura, and W. J. Harrington, *Blood*, **15**, 388 (1960).

[4] Nakeff, A., and M. Ingram, Atomic Energy Project, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y. 14620. Personal communication.

[5] Aster, R. H., and J. H. Jandl, *J. Clin. Invest.*, **43**, 843 (1964).

[6] Cassidy, J. T., G. W. Jourdian, and S. Roseman, in *Methods in Enzymology*, ed. E. F. Neufeld and V. Ginsburg (New York: Academic Press, 1966), vol. 8, p. 680.

[7] Warren, L., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1963), vol. 6, p. 463.

[8] Popenoe, E. A., and R. M. Drew, *J. Biol. Chem.*, **228**, 673 (1957).

[9] Tocantins, L. M., *Arch. Pathol.*, **21**, 69 (1936).

[10] Gottschalk, A., in *The Amino Sugars*, ed. E. A. Balazs and R. W. Jeanloz (New York: Academic Press, 1966), vol. 2B, p. 337.

[11] Marcus, A. J., and M. B. Zucker, *The Physiology of Blood Platelets* (New York: Grune and Stratton, 1965), pp. 49, 59, and 72.

[12] Luscher, E. F., *Brit. J. Haematol.*, **13**, 1 (1967).

[13] Weiss, L., *The Cell Periphery, Metastasis, and other Contact Phenomena* (New York: John Wiley and Sons, 1967), p. 324.

[14] Cronkite, E. P., V. P. Bond, T. M. Fliedner, D. A. Paglia, and E. R. Adamik, *Blood Platelets* (Boston: Little, Brown and Co., 1961), p. 595.

[15] Mustard, J. F., H. C. Rowsell, E. A. Murphy, and M. F. Glynn, *Blood*, **25**, 613 (1965).

[16] Anderson, B., and T. T. Odell, *Proc. Soc. Exptl. Biol. Med.*, **99**, 765 (1958).

[17] Gore, I., M. Takada, and J. Austin, *Federation Proc.*, **27**, 672 (1968).