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*REMOVAL OF SIALIC ACID FROM THE CELL COAT IN TUMOR CELLS AND VASCULAR ENDOTHELLUM, ANDITS EFFECTS ON METASTASIS**

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The fate of circulating neoplastic cells depends primarily on their adhesiveness with respect to the vascular endothelium. If they adhere, metastases may be formed.

It is very likely that the capacity of circulating tumor cells to adhere to vascular endothelia must be greatly influenced by the sticky properties of both surfaces which make contact during the process of metastatic tumor formation, although this has not yet been fully established by *in vivo* experiments. A possible variation of the stickiness in the vascular lining has already been suggested by Zeidman,[1] who has observed that mesenteric capillaries in cortisonized rabbits trapped more tumor cells than similar capillaries in normal controls. But changes in the coat of tumor cells, as shown by the Hale staining technique,[2] may be equally important in modifying their stickiness. In our experience (unpublished), the ability of intravenously injected mouse ascitic cells to produce lung tumors seems to be related to the existence of a good mucopolysaccharide coating. However, other properties of this coat, still to be determined, may also play an important role.

In view of the fact that the Hale positive component of the coat in mouse ascitic tumor cells, a sialomucin in nature,[3] as well as the Hale positive coat of the vascular lining (see below), may be removed by the receptor-destroying enzyme (RDE), experiments were performed to determine whether the enzymatic treatment of these two types of cells may affect the frequency of metastases. For this purpose, a

commercial preparation of RDE from *V. cholerae* (Behringwerke Ag., Marburg-Lahn, Germany) was used, dissolving the contents of 1 vial in 2.5 ml of Tris-maleate buffer at pH 5.6 (300–500 units per ml).

In three experiments, A/sn or CAF1/J mice, eight weeks old, were inoculated by the way of their tail veins with uncoated cells from the mouse ascitic tumor TA3. These cells, before being injected, were washed and resuspended in basal Eagle's medium and then incubated at 37°C for 1 hr, with slow stirring, in the presence of RDE (1 or 2 drops of the enzyme solution per ml of Eagle's medium containing 10^6 cells). After this treatment, the cells were washed again and resuspended in the same nutritive medium. Each mouse received 200,000 or 350,000 cells in 0.20 ml of an ice-cooled suspension, kept uniform by slow stirring. Control animals received the same number of untreated TA3 cells. Mice from both groups were killed two weeks later and lung metastases counted.

In spite of the fact that RDE was active, *in vitro*, in removing partially or completely the Hale positive material of the tumor cell coat (Figs. 5 and 6), no difference in metastatic spread was observed when mice were injected with coated or uncoated cells (Table 1).

TABLE 1

LUNG TUMORS IN MICE INOCULATED WITH COATED OR UNCOATED TA3 CELLS

Type of inoculation	No. of mice	Mean no. of tumors counted	No. of mice with tumors	Diameter of largest tumors mean and range (mm)
Coated	60	7.08	56	1.5 (0.5–3.0)
Uncoated	60	6.83	58	1.9 (0.5–5.0)

Difference between means = 0.25. Standard error of difference = 1.30. *t* difference = 0.19. *P* difference > 0.05.

This result was not wholly unexpected. It was already known that enzymatically uncoated TA3 cells can regenerate *in vitro* their Hale positive coat within 2 hr if cultivated in an appropriate medium.[3] It is very likely that the same phenomenon may occur *in vivo*, explaining why a previous removal of the coat in TA3 cells is without effect on their metastatic behavior. It is quite probable that these cells regenerate their sticky coats *in vivo*.

In the second part of this work, an attempt was made to study the role of the vascular endothelial surface on metastases by experiments in which this coat was modified by RDE *in vivo*. However, it was first necessary to investigate the toxicity of this enzyme and how it acts on blood vessel linings. It was established that eight-week-old mice were able to withstand an intravenous injection of 0.20 ml of the enzyme solution without showing visible signs of toxicity or loss of weight. No tests were carried out, however, with greater doses. When 0.20 ml of RDE was given intravenously and treated mice, together with the appropriate controls, were killed at different intervals, it was found that this enzyme removed the Hale positive coat from the lung or liver vascular endothelium. The removal was almost complete at 3 hr (Figs. 1 and 2), and the effect lasted for 18 hr or more (Figs. 3 and 4). Full recovery of the coat in lung blood vessels did not take place until four days later. The action of RDE was not limited to the vascular lining of the lungs. RDE treatment also affected extravascular sites such as the endothelial linings of bronchial tubes and lung alveoli (as illustrated in Figs. 3 and 4). It did not affect the Hale-positive intracellular cytoplasmic granules of certain interstitial cells

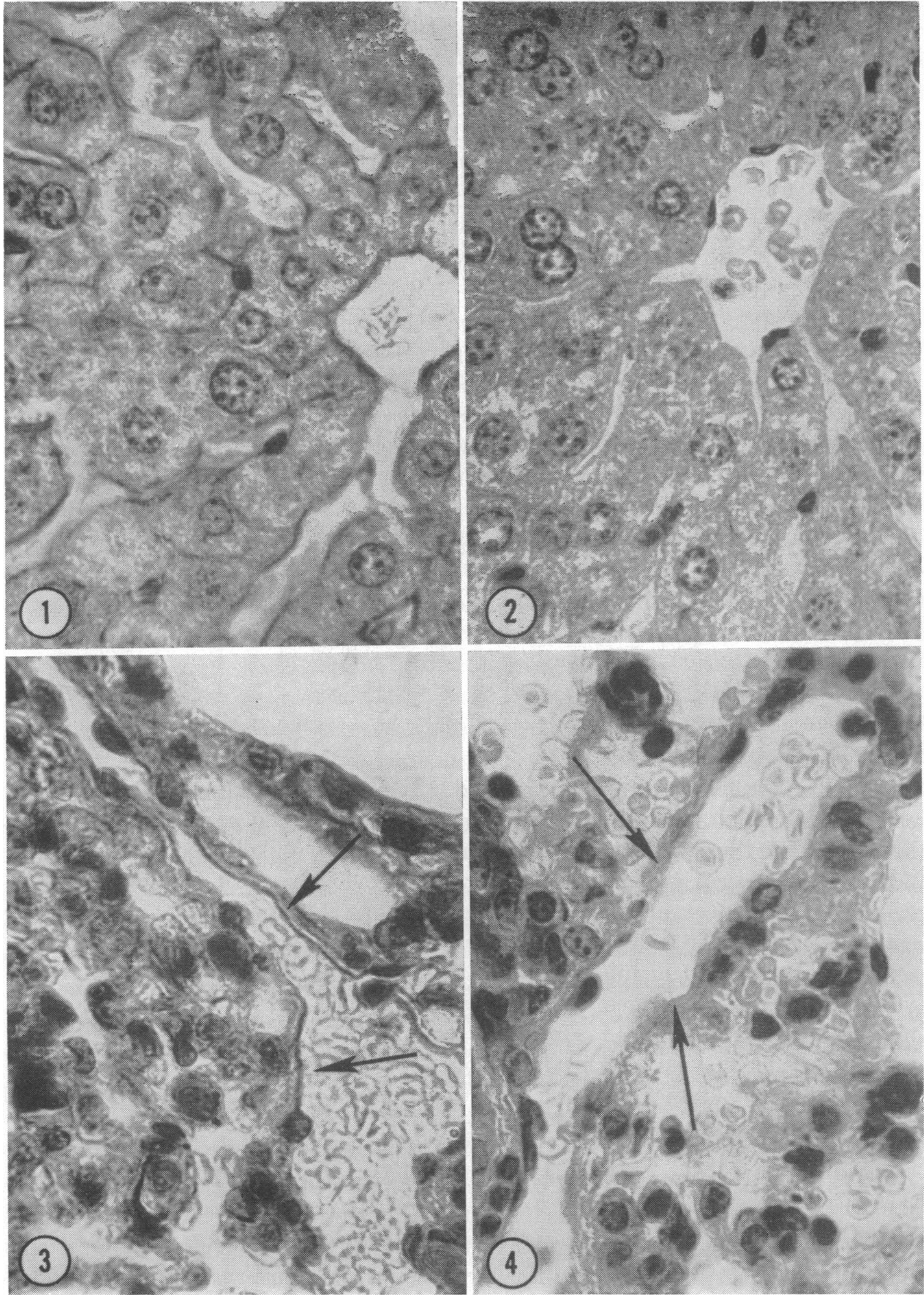

FIG. 1.—Liver (control): A Hale positive substance in the lining of the vascular surface ×750.
FIG. 2.—Liver (3 hr after RDE injection): Removal of this lining ×750.
FIG. 3.—Lung (control): Arrows show a Hale positive vascular coating ×850.
FIG. 4.—Lung (18 hr after RDE injection): Complete removal of this coating. Fixative: Formalin-sublimate. Nuclear counterstaining with haemalum.
(Photographs by William Fore.)

of the lung. And it should be noted that these cells seem to increase in number in treated animals.

Pure neuramidase in Tris-maleate buffer of pH 7.0 (1 mg of protein per ml) was also tested *in vivo*, and it proved to be even more active than RDE.

After these preliminary trials, three experiments were performed. Treated mice from each experiment received intravenously 0.20 ml of the RDE solution. Controls were injected with the same amount of the buffer. At different intervals after enzymatic treatment (1, 5, and 18 hr), both groups were inoculated by way of the tail vein with 0.20 ml of a cell suspension in basal Eagle's medium, containing approximately 150,000 or 200,000 of untreated TA3 cells, previously washed with the same fluid. To keep constant the number of injected cells, the ice-cooled suspension was stirred slowly with a magnetic stirring device. During tumor inoculation, one treated mouse was alternated with one control. Two weeks later, the mice were killed and lung metastases counted with a dissecting microscope. Metastases in other sites were also recorded.

When the data of the three experiments were pooled and submitted to statistical analysis, the difference between treated and untreated mice was significant (t diff. = 6.64, p diff. < 0.001) (Table 2). Metastases were also observed in sites other than the lung and they were less frequent in treated animals (1 out of 50 against 6 out of 49).

Since a sample of pure neuramidase is as active or even more active than RDE, it is believed that neuramidase is the enzyme responsible for the removal of the Hale positive material, a sialomucin in nature. Though part of the RDE must cross the blood vessel barrier to act in other sites, the rest must stay in circulation, presumably, for 24 hr or more, thus explaining the long effect on the vascular endothelium, unless a slow regeneration of the coat, after one enzymatic attack, is

TABLE 2

LUNG TUMORS IN MICE TREATED OR UNTREATED WITH RDE BEFORE INOCULATION WITH COATED TA3 CELLS

Exper. group	No. of mice	Mean no. of tumors counted	No. of mice with tumors	Diameter of largest tumors, mean and range (mm)
Untreated mice	49	6.82	47	1.4 (0.4–2.5)
RDE-treated mice	50	1.84	28	1.1 (0.2–2.0)

Difference between means = 4.98. Standard error of difference = 0.75. t difference = 6.64. P difference $<$ 0.001.

the correct interpretation. It is interesting to note that RDE, *in vitro* and above pH 7, is a highly stable enzyme.[6] On the other hand, it has been reported that it is not antigenic,[7] a property that may be of use if repeated injections of RDE were required.

In spite of the fact that we cannot exclude other mechanisms of action, because RDE is also very active on other structures of the host, we believe that the antimetastatic effect of this enzyme is a phenomenon connected with the removal of sialic acid from the surface making contact. However, we are not yet in a position to say whether the reduction in the number of metastases is due exclusively to action of the enzyme on the vascular coat or to an influence both on this coat and on the surface of the tumor cells in circulation.

It is assumed that the removal of sialic acid from the rest of the mucopoly-

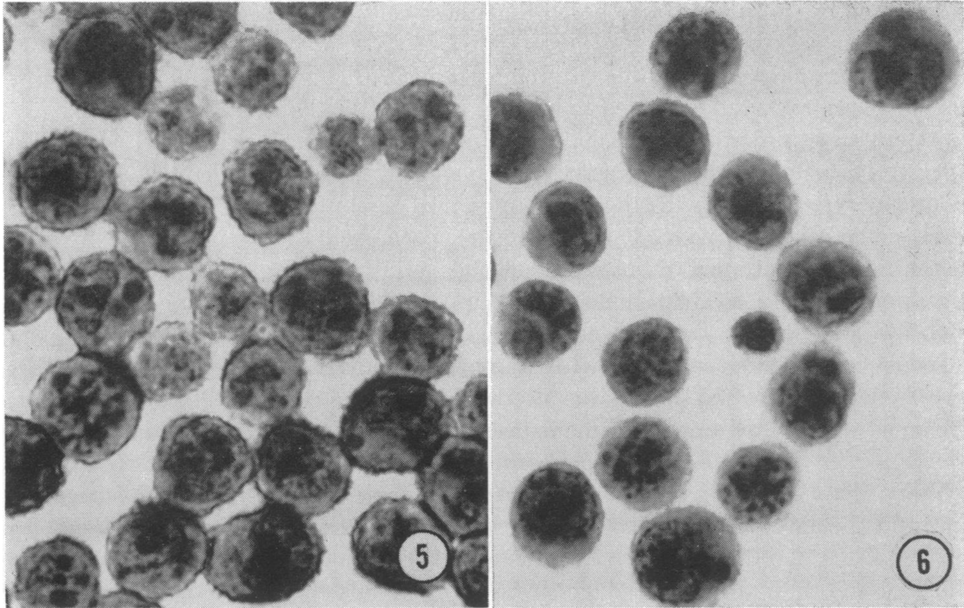

FIG. 5.—Thick smear cf untreated TA3 cells showing a Hale positive coating ×850.
FIG. 6.—Thick smear of RDE-uncoated TA3 cells ×850. Fixative: Formalin-sublimate. Nuclear counterstaining with haemalum.
(Photographs by William Fore.)

saccharide polymer reduces cell stickiness between circulating tumor cells and the vascular lining. Though not the same process, it is already known that similar enzymatic treatment diminishes or suppresses adhesiveness of cells to a glass surface.[4, 5]

In general, it is our impression that tumors in treated mice were smaller. One reason for this may be that RDE treatment delays the time of implantation.

Among other explanations to be entertained is a toxic effect of RDE acting either on tumor cells or upon the host. We believe that both of these actions could be ruled out because *in vitro* treated cells grow well when implanted subcutaneously and treated inoculated hosts do not loose weight or very little at the end of the experiments.

To establish whether a treatment with a neuramidase-containing preparation may be a more rational approach to the practical problem of controlling tumor spread, further research will be needed to see whether under other experimental conditions (stronger or repeated doses of the enzyme) better results may be obtained and whether this treatment may be equally effective against metastases produced by other type of malignancies.

Summary.—It is very likely that adhesiveness between circulating neoplastic cells and vascular endothelium is a phenomenon depending on the sticky properties of both surfaces which make contact during the process of metastases formation. On the hypothesis that this stickiness may be produced by mucopolysaccharides, several experiments were performed to determine whether neuramidase-containing preparations, which remove sialomucins from the cell surface, may affect metastatic spread. No modification was found when *in vitro* RDE-uncoated tumor cells were

inoculated intravenously. However, when the enzyme was injected by the same route before inoculating coated tumor cells to homologous hosts, a significant decrease of metastases was observed.

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*RESTING AND ACTION POTENTIAL OF INTRACELLULARLY PERFUSED SQUID GIANT AXON**

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There has been in the past very extensive work dealing with the effects of varying the chemical composition of the external fluid medium upon the resting and action potentials of excised axons. Based on the analyses of these effects, the nature of the resting membrane potential and the mechanism of nerve excitation have been discussed (see, e.g., Grundfest[1]). It is highly desirable, in order to clarify the excitation mechanism, to investigate the effects of altering the chemical composition of the intracellular phase as well as that of the extracellular fluid medium. However, it was not possible, until quite recently, to drastically alter the chemical composition of the interior of an axon without rendering the axon inexcitable.

Following various experiments involving massive injections of various solutions into squid giant axons, it became increasingly clear that squid axons can withstand a procedure of continuous intracellular perfusion without losing their ability to carry nerve impulses (see Oikawa, Spyropoulos, Tasaki, and Teorell[2]). In a recent communication, Baker, Hodgkin, and Shaw[3] reported their success in perfusing squid axons and described the effects of various kinds of perfusing fluid upon the electrophysiological properties. Using radioactive tracers in conjunction with perfusion, the permeability of the axonal membrane to various ions was investigated.[4] Davies[5] reported the feasibility of perfusing the interior of the frog muscle fiber.