Removal of PAS Positive Surface Sugars in Tumor Cells by Glycosidases.* (28762)

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Structural components of the coat of free tumor cells, particularly from epithelial malignancies, may be stained both by the Hale and the PAS procedures(1). The Hale positive material can be removed by *active* neuramidase, indicating the presence of sialic acid. The PAS positive component is not influenced by lipid extraction and diastase digestion, excluding lipids and simple polysaccharides. This leaves simple sugars of the neutral mucins(2) of the coat or neuraminic (sialic) acid(2,3) as the most likely PAS positive components. On testing the participation of sialic acid, we found that treatment with a potent neuramidase from *Streptomyces albus* only slightly diminished the intensity of the PAS reaction in the cell coat. Therefore, simple sugars of the neutral mucins are probably the main PAS positive material of the cell surface.

Sugars or amino sugars are known to be the basic components of the blood group substances and specific sugars of these blood group substances can be split off by specific glycosidases from *Clostridium* perfringens or *Trichomonas foetus,* a treatment that changes their serological specificity(4). We performed analogous experiments with glycosidases, to test our cytochemical interpretations and to investigate which sugars of the tumor cell coat are responsible for its PAS positive reaction.

Methods. TA3 cells from a 4 days old ascites tumor were used throughout the work. This tumor originated in the mammary gland of a female mouse from strain A(5). TA3 cells were washed with a culture medium without serum (Eagle or NCTC-109), fixed for one hour in a formalin-mercury chloride solution(6), and paraffin embedded for sectioning at 3 μ. After removal of mercury and before being stained with a Hale iron technique(7) or the PAS method(7), sections were subjected to lipid extraction or enzyme incubation.

Lipids were extracted for 24-48 hours by using 95% phenol(4) or a chloroform-methanol solution at 60°C. Sections were incubated at 37°C in the presence of one of the following enzymes: 1) malt diastase (Nutritional Biochemicals Corp.)—50 mg per 50 ml of 0.1 M phosphate buffer, pH 6, containing 0.4% NaCl; 2) receptor destroying enzyme (RDE) from *Vibrio cholerae* (Behringwerke Ag., Marburg-Lahn, Germany)—usually 4 I.U. of neuramidase per 0.1 ml of 0.1 M acetate buffer, pH 5.6, containing 0.04 M CaCl 2; 3) neuramidase from *Streptomyces albus,* generously supplied by Dr. Morris Zimmerman, Merck & Co., Rahway, N. J.—100 μg of protein per 0.1 ml or 0.1 M acetate buffer, pH 5.6, containing 0.04 M CaCl 2; 4) a partial purified preparation of glycosidases from *Clostridium perfringens,* kindly provided by Dr. Bertram Gesner and Dr. Victor Ginsburg, Nat. Inst. Health, Bethesda—1 part of the enzyme solution** (partially inactivated at 45°C for 10 minutes) and 9 parts of 0.1 M phosphate buffer, pH 7.2, or 0.1 M acetate buffer, pH 5.6, and 5) a highly purified preparation of N-acetylhexosaminidase from fungi of the *Chalaropsis* genus(8), obtained through the courtesy of Dr. John Hash, Lederle Laboratories, Pearl River, N. Y.—50-100 μg per 0.1 ml of 0.1 M acetate buffer, pH 5.6. Control sections were incubated with the buffer alone or with the heat-inactivated enzymes. (Neuramidase was heated in a water bath at 55°C for 30 minutes, in the absence of calcium(9). Glycosidases were inactivated at 65°C for 15 minutes.) The incubation times for diastase, neuramidases, and glycosidases were 1, 5, and 18 hours, respectively.

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** 2.4 mg protein/ml.

The activity of neuramidase was blocked by adding to 0.1 ml of the enzyme solution 2 mg of neuraminic acid (California Corp. for Biochemical Research) or 2 mg of a hydrochloride and amino derivative of 2-phenyl-3beta-chloroethyl-4-keto-2,3,5,6-tetrahydro-oxazyne (SIMO SOLUBLE), a sialic acid analog(10), generously supplied by Vittorio Molina de Gaudenzio Chemical Industry, Novara, Italy. To block specifically one of the glycosidases from *Cl. perfringens*(4,11), 3 or 6 mg of one of the following sugars, obtained from Mann Research Laboratories, were added to 0.1 ml of the enzyme solution: L-Fucose (6-Deoxy-L-Galactose), D-Galactose, D (+) Mannose, A-Acetyl-D-Glucosamine, and N-Acetyl-D-Galactosamine. In some experiments a mixture of the 5 sugars (3 mg of each one) was tested. After incubation, sections were carefully washed with distilled water.

Results and discussion. We observed in several experiments that the mixture of enzymes from *Cl. perfringens* was active against the PAS positive component of the tumor cell coat. When similar sections were incubated with buffer or heat-inactivated enzymes, this component was unaffected (Fig. 1 and 2). The activity of the preparation could, therefore be attributed to its content of glycosidases.†

To determine which of the glycosidases was responsible for elimination of the PAS positive component, sections were incubated in the usual medium, to which a single and different sugar was added each time, for the purpose of blocking a specific glycosidase(4, 11). In addition, the mixture of 5 sugars was also tested. It was found that this mixture was very effective in preventing removal of the PAS positive material. Among single sugars, acetyl-galactosamine and galactose were almost as active as the mixture of all five. Fucose was intermediate and acetyl-glucosamine and mannose were the least active. This suggests either that acetyl-galactosamine and galactose are the more frequent PAS positive repeating units or that removal

† The preparation of *Clostridium* glycosidases also showed neuramidase activity because this split off the Hale positive component. The neuramidase activity could be prevented by the addition of neuraminic acid or SIMO SOLUBLE to the incubation medium.

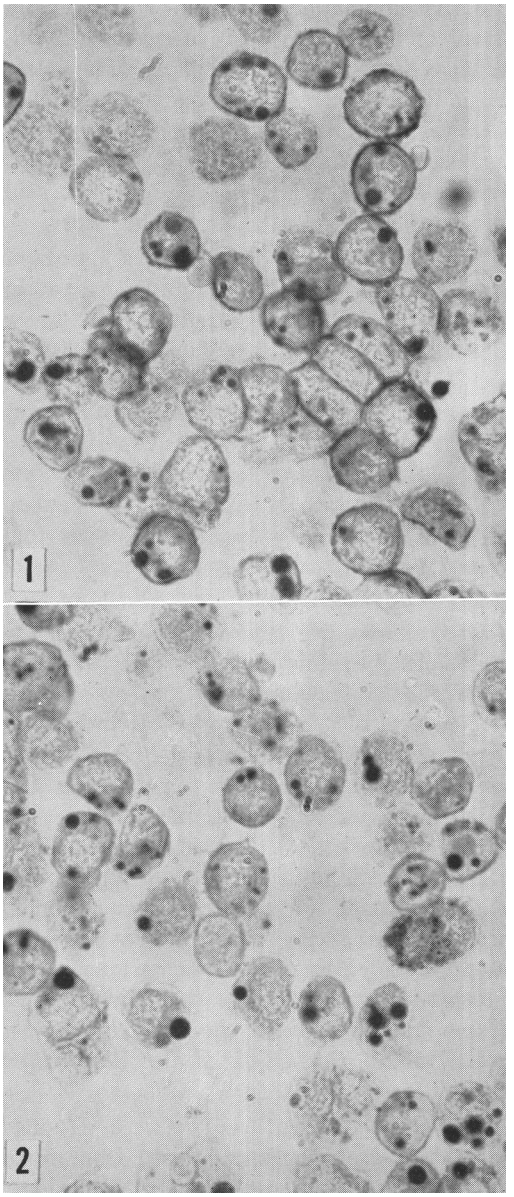

FIG. 1. Section of TA3 tumor cells incubated with acetate buffer, pH 5.6, and stained with PAS method. Most cells show coats with an intense PAS positive reaction. Cytoplasmic bodies of variable size are also PAS positive. × 936.

FIG. 2. Section of TA3 tumor cells incubated with Clostridium glycosidases in acetate buffer. This treatment removes most of PAS positive material from cell coat. Cytoplasmic bodies are unaffected or very little modified. × 936.

of a few units of these sugars releases shorter polymers containing other PAS positive sugars which can then be washed away.‡ Fucose appears to be a side or terminal residue (its elimination not resulting in the removal of other PAS positive sugars) or, alternatively, to be less abundant. The latter possibility would be in agreement with biochemical data of other authors(13), who by analysis of different types of glycoproteins have found that a high amount of sialic acid correlates with a low quantity of fucose.

Though we do not have information about the sequence of sugars or amino-sugars in the PAS positive surface polymer, we know that sialic acid, a Hale positive substance, is terminal. This can be inferred from the fact that the PAS positive coat is modified very little by neuramidase treatment, and, if the same TA3 cells are stained first with the Hale stain and then with the PAS method (7), the cells appear to have an inner thin layer of PAS positive material and outer layer of thicker Hale reactive substance.

Glycosidases are enzymes capable of hydrolyzing glycosidic bonds. Some may act on terminal linkages; others may split bonds at any level of the multisugar polymer. Glycosidases from *Cl. perfringens* seem to belong to the first group, acting only on terminal linkages, as (when neuramidase impurity is blocked): 1) the glycosidases do not split PAS positive sugars if neuraminic acid, known to be a terminal molecule, is present; and 2) they do not alter the Hale staining under the same circumstances. An alternative explanation is that the presence of neuraminic acid in the surface polymer might inhibit the action of the *Clostridium* glycosidases.

Somewhat different results were obtained using a N-acetyl-hexosaminidase from fungi of the genus *Chalaropsis*(8). This glycosidase can remove the PAS positive material although the Hale positive component is only partially removed. When blocking agents of neuramidase are used, *Chalaropsis* glycosidase is still active, indicating that the action of this enzyme is independent of the presence or absence of neuraminic acid. Another possible interpretation of the persistence of the Hale staining when *Chalaropsis* enzyme is used alone is that this treatment may uncover additional Hale reactive material after removal of the surface sialomucins. The *Chalaropsis* enzyme is also different from the *Clostridium* preparation in that it is not blocked by the 5 sugars, singly or together.

We do not know whether there is a relationship of the surface sugars in TA3 cells with those of the blood group substances known to be present in different types of cells and secreted by mucous cells(14). Also, we do not know whether the identified sugars might be specific for our tumor cells or for the tissue from which TA3 cells originated, all points of great biological interest. Perhaps sugars of the cell coat might be important for specific cell aggregation. In this connection, experiments with rat lymphocytes, done by Gesner and Ginsburg(11), should be mentioned. These authors have observed that *in vitro* treatment of lymphocytes with *Clostridium* glycosidases prevents their selective accumulation in lymphoid tissues when injected into the circulation. They have suggested that this treatment might destroy the specific polysaccharide surface structure of the lymphocytes, which is normally recognized by complementary structures in the lymphoid tissue.

Summary. Structural components of the coat of fixed TA3 tumor cells are stained both by the Hale and the PAS procedures. The Hale positive material can be removed by active neuramidase, indicating the presence of neuraminic acid. The PAS positive component is not influenced by lipid extraction or by diastase digestion and is only slightly affected by neuramidase. However, it is strongly affected by an enzyme preparation from *Clostridium perfringens,* containing a mixture of glycosidases. By specific blocking of individual glycosidases in the incubation medium, it was determined that galactose and acetyl-galactosamine are im-

‡ If, as reported by Hooghwinkel and Smits for hexosamines(12), acetyl-galactosamine is not oxidized by periodic acid, the first alternative can be excluded for this sugar.

portant components for the PAS positivity of the cell coat. Fucose is of less significance and perhaps less abundant than other PAS positive sugar units. N-acetyl-hexosaminidase from fungi of Genus *Chalaropsis* was also active against the PAS positive cell coat but differed from the glycosidases of *Cl. perfringens* in that it did not require the previous removal of the Hale positive component and was not blocked by N-acetyl-galactosamine, galactose, and fucose.

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Electron Microscopic Observations of Simian Virus-40 in Primary Rhesus Kidney Tissue Cultures. (28763)

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Electron microscopic observations of simian virus 40 (SV-40)(1) have been made in kidney cell cultures from cercopithecus(2,3, 4), and erythrocebus(5) monkeys and have contributed to an accurate description of the cytoplasmic vacuolization which is the characteristic cytopathic effect of SV-40 in these cell systems. Although SV-40 also multiplies readily in rhesus kidney cell cultures, it produces no distinct cytopathic effect and ordinarily may be detected only by subculture in cercopithecus kidney cells. Shein and Levinthal(6), however, using fluorescein-tagged antibody, were able to find SV-40 antigen in rhesus kidney cell cultures. Particles of this virus may also be seen in rhesus kidney cells by direct observation with the electron microscope, as described in the present report.

Materials and methods. Monolayer tissue cultures of primary rhesus kidney were prepared in 2-ounce prescription bottles. The cells were maintained in weekly changes of medium 199 plus 2% calf serum; each bottle contained 6 ml of medium. Experimentally infected tissue cultures were given $10^{5.8}$ $TCID_{50}$ per bottle of a subpassage of SV-40 strain 776(1); the inoculum was contained in 0.1 ml. BS-C-1 cell cultures(7), which were derived from cercopithecus kidney, were used for SV-40 titrations. The cells were held for 28 days, with weekly medium changes. Both the rhesus and the BS-C-1 cells were kept at 36°C.

For electron microscopy, rhesus cells were removed from the bottles, fixed in 1% OsO_4, dehydrated in graded alcohols, embedded in 1:4 methyl:butyl methacrylate, sectioned on a Porter-Blum ultramicrotome, and observed, unstained, in a Philips model 100B electron microscope.

Results and discussion. Cells in the first