HALE STAIN FOR SIALIC ACID-CONTAINING MUCINS

Adaptation to Electron Microscopy

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ABSTRACT

The feasibility of using the Hale stain to identify cellular sialic acid-containing mucins by electron microscopy was investigated. Three kinds of mouse ascites tumor cells were fixed in neutral buffered formalin, exposed to fresh colloidal ferric oxide, treated with potassium ferrocyanide, imbedded in Selectron, and sectioned for electron microscopy. Additional staining with uranyl acetate and potassium permanganate was done after sectioning in order to increase contrast. Those cells known to be coated with sialomucin showed deposits of electron-opaque ferric ferrocyanide crystals in the areas where sialomucin concentrations were expected. When these cells were treated with neuraminidase beforehand, these deposits did not appear. It was concluded that, with the precautions and modifications described, the Hale stain can be successfully combined with electron microscopy to identify sialomucin.

INTRODUCTION

It has been reported that cells from certain ascites tumors are surrounded by a Hale-positive coat (1), and that the material responsible for this reaction is a sialomucin (2, 3), one of the acid mucopolysaccharides. The thickness of this coat when stained by the Hale method and examined with the light microscope may vary considerably, depending on the type of tumor cell. Before investigating the causes of these variations and their meanings, an attempt was made to determine whether the Hale procedure could be adapted to electron microscopy. If successful, this would permit the study of sialomucins at a level beyond the reach of ordinary microscopy.

MATERIALS AND METHODS

Three kinds of mouse ascites tumor cells, differing in the thicknesses of their sialomucin coats, were selected for investigation. These were: (*a*) TA3 cells from a mammary adenocarcinoma, shown to have a thick coat of this material by the usual histological techniques (1–3); (*b*) MC1M sarcoma cells, shown to have practically no sialomucin coat (1); and (*c*) 15091A cells from an undifferentiated mammary adenocarcinoma, shown to have a moderately thin sialomucin coat (1).

The cells were collected directly from the abdominal cavities of the hosts and washed twice with Eagle's basal medium to remove possible contamination by serum, which is known to contain sialomucin (4, 5).

The cells were fixed by a variety of methods. The oxidizing fixatives, such as osmium tetroxide and potassium permanganate, interfered with the later staining reactions and could not be used. The reducing fixatives, such as alcohol and formaldehyde, were suitable, at least to the extent that there was no interference with the desired reaction. Unfortunately, these restrictions in the choice of fixatives resulted in the usual limitations of fine structural morphology.

In general, the cells were fixed overnight at room temperature in 10 per cent neutral phosphate-buffered formalin. This was found to give better structural detail than fixation with Love's sublimated formalin ordinarily used for the Hale method (6).

After a number of washings with water, the cells were stained while still in suspension, using Mowry's modification of the Hale method (7). This involves the use of fresh colloidal ferric oxide prepared according to Müller as described by Mowry (7). Briefly, the cells were rinsed in 12 per cent acetic acid, exposed to fresh Müller's colloidal iron for 60 minutes, rinsed 4 times in 12 per cent acetic acid, treated for 20 minutes in a fresh mixture of 2 per cent hydrochloric acid and 2 per cent potassium ferrocyanide, and finally rinsed 3 times in distilled water. No counterstain was used. It is emphasized that Mowry's recommendation for limiting the pH of the colloidal iron solution to 1.1 to 1.3 was strictly followed, since this minimizes the non-specific staining of nucleic acids. As a further precaution, to insure that naturally occurring iron was not mistaken for sialomucin, controls were used. These were treated exactly as described above except for exposure to the colloidal iron.

In order to make certain that the stained material was truly a sialomucin, cells were treated with neuraminidase before staining.

In this procedure, TA3 cells were suspended in the chemically defined medium NCTC-109 and incubated at 37°C for 4 hours in the presence of purified neuraminidase (*Vibrio cholerae*).[1] To each ml of cell suspension containing 2×10^6 tumor cells was added 100 micrograms of enzyme protein. The cells were then washed and fixed with formalin as described above. A control group of similar cells was incubated without the enzyme.

In addition, similar cell suspensions were incubated in the presence of inactivated neuraminidase to insure that non-specific protein masking was not taking place (8, 9). Neuraminidase in calcium-free water was inactivated by heating at 55°C for 30 minutes (10).

Several types of embedding media were investigated, including methacrylates, epoxies, and polyesters. In the experiments reported here, the method finally adopted was that of Low and Clevenger (11) which utilizes a polyester. However, the recommended heating after sectioning was omitted, since the high temperatures affected the desired crystal formations. This material, like the epoxies, has a low inherent contrast but, by appropriate staining methods after sectioning, the contrast can be enhanced considerably. Although the crystals which were formed were insoluble in water, there was a tendency to promote their floating and deposition in secondary sites after too long an immersion in the staining solution. Consequently, the staining time was held to a minimum. Our best results were obtained by exposing the sections to a bath of 1 per cent potassium permanganate for 15 seconds, followed by a quick rinse in distilled water, and a second exposure to 1 per cent uranyl acetate for 15 seconds, again followed by a few quick water rinses and final drying.

[1] The purified neuraminidase was supplied by Dr. Leonard Warren, National Institutes of Health, Bethesda, Maryland.

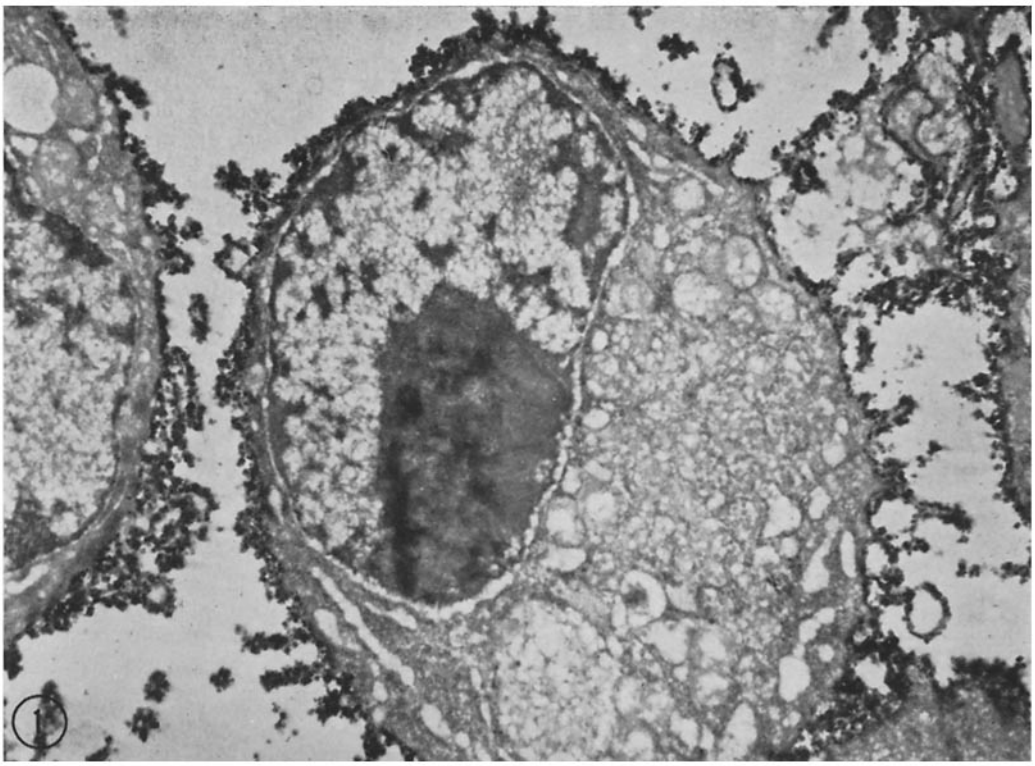
RESULTS

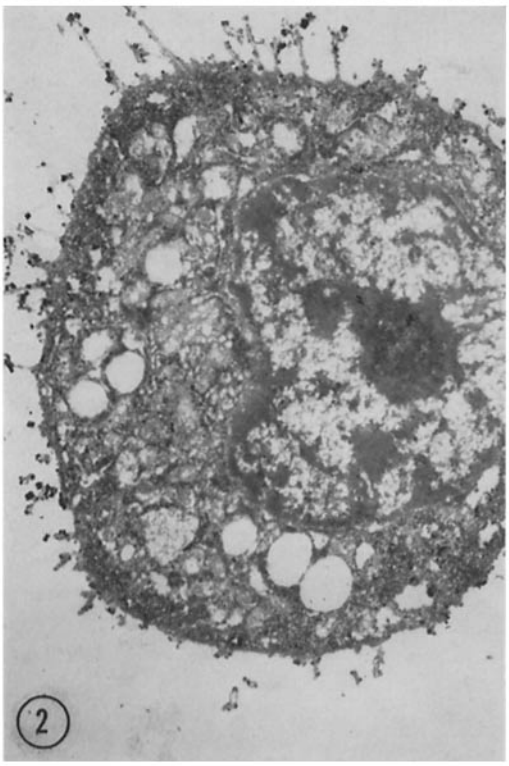
The usefulness of the Hale stain depends on the formation of ferric ferrocyanide where sialomucins exist. This is seen as a blue mass by ordinary light microscopy. With the higher resolution of the electron microscope, these masses appear as distinct crystals and aggregates of crystals of ferric ferrocyanide. On theoretical grounds, it should not be necessary to use the complete Hale stain since the blue color of the ferric ferrocyanide which is essential to light microscopy is of no value in electron microscopy. However, if the procedure is stopped after exposure to the colloidal iron, the resulting sections are difficult to interpret. The bound colloidal iron consists of extremely small particles which not only require very high magnification but also are easily confused with bodies of similar size of non-specific nature. With the use of the complete Hale stain, however, one gets definite crystal formations of ferric ferrocyanide which are much larger than the particles of colloidal iron and are quite different from the non-

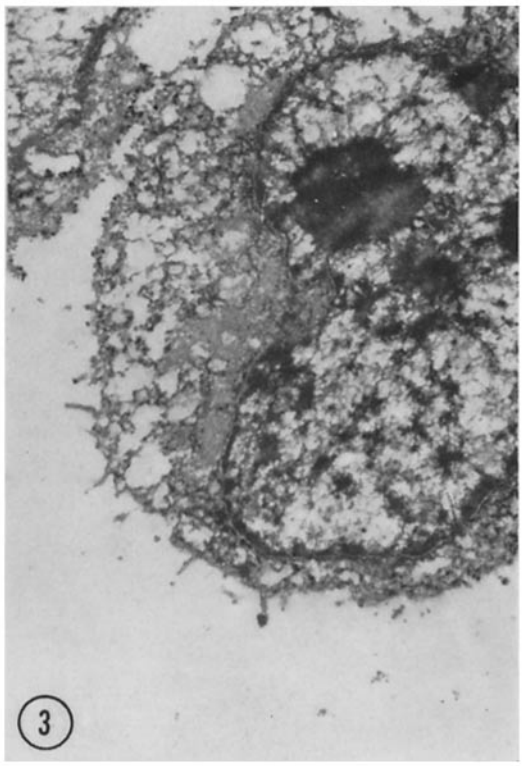
FIGURE 1 Section of mouse ascites tumor cells TA3 stained by the modified Hale method. Crystals of ferric ferrocyanide coat the surfaces and outline the fine processes. × 15,000.

FIGURE 2 Section of a mouse ascites tumor cell 15091A stained by the modified Hale method. The paucity of crystals reflects the greatly decreased concentration of sialomucin in the cellular coat. × 15,000.

FIGURE 3 Section of mouse ascites tumor cells MC1M stained by the modified Hale method. These cells are known to have almost no acid mucopolysaccharides in their coats. × 15,000.







crystalline structures. This is shown in Fig. 1, a thin section through a TA3 cell stained by the modified Hale method. The Hale-positive material appears as a surface coat of electron-opaque crystals and crystalline aggregates. Individual crystals range in size from 0.035 to 0.085 micron, too small to be seen by ordinary light microscopy. Some aggregates, of course, are large enough to be visualized with the light microscope, but at best, one sees with this technique a coarse mass with no structural detail. The cell nucleus and the cytoplasmic structures are sharply defined by the supplementary stains. These are amorphous, however, and are clearly different from the fine crystal formations.

The cellular cytoplasm appears vacuolated. Most of these "empty" structures are mitochondria from which the cristae have been leached. They can be identified easily by their characteristic internal structure when osmium tetroxide is used for fixation instead of formalin (Fig. 7). Occasionally, some pinocytotic vacuoles are present which give a Hale-positive reaction (Fig. 5). However, a more complete review of the Hale-positive cytoplasmic organelles will be considered in future publications.

Examples of other ascites tumor cells are shown in Figs. 2 and 3, tumors 15091A and MC1M respectively. These have been included to demonstrate differences in the sialomucin coats of various cells. Tumor 15091A has a decidedly weaker reaction to the Hale stain than TA3 cells, and tumor MC1M has practically no sialomucin in its coat.

When the cells are not exposed to colloidal iron, no crystals are formed (Fig. 6). It is advisable to carry such a comparison series when using the Hale technique, since there is always the possibility of naturally occurring ferric iron.

When TA3 cells are treated with neuraminidase, practically all evidence of sialomucins in the coat disappears and the material becomes Hale-negative (Fig. 4). The effect of the enzyme is readily apparent when the treated cells are compared with the controls (Fig. 5). The heat-inactivated enzyme had no such effect, leaving the Hale-positive coat intact.

DISCUSSION

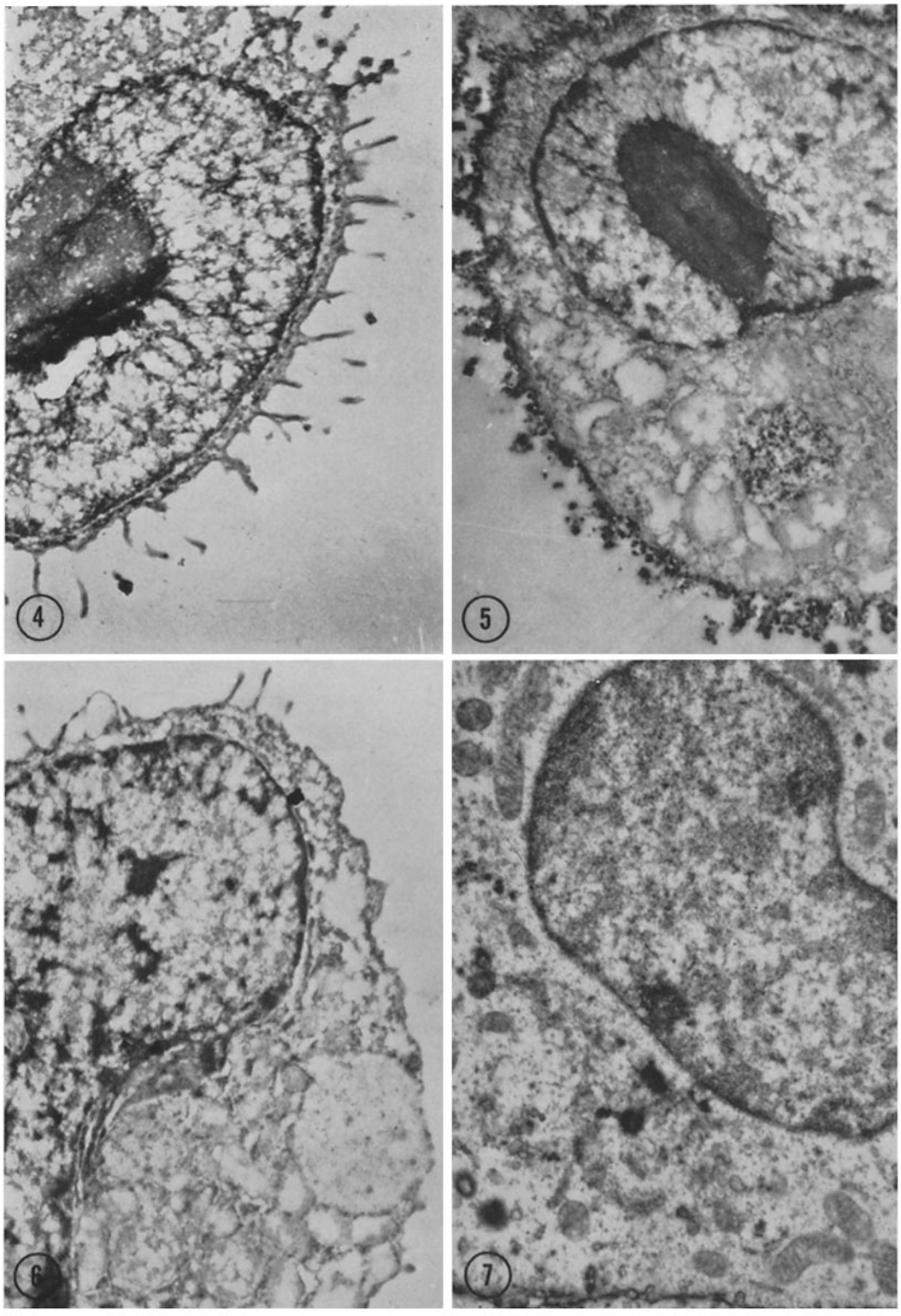
Since its introduction in 1946, Hale's method for acid mucopolysaccharides in general has been both criticized and defended by various workers (12). The method has been modified and improved by a number of investigators, principally by Mowry (7). By the use of the specific enzyme neuraminidase, one can identify sialic acid-containing mucopolysaccharide. When the Hale reaction is positive, a precipitate appears at the external cell surface. This precipitate, which is ferric ferrocyanide, does not appear unless the cell is first exposed to iron which combines with available free acidic groups. This reaction is abolished by the enzyme neuraminidase. Since this enzyme is considered specific for the sialic acid-containing mucopolysaccharides, it seems reasonable to assume that the crystal formation, at least at the external surface of the cell, is associated with sialomucin. The situation with respect to the internal structures is not quite so clear. The control cells of the enzyme experiment (Fig. 5) show scattered crystal formations which cannot be identified with specific structures with any certainty. Moreover, these formations persist in the enzyme-treated cells even though the coat

FIGURE 4 Section of a mouse ascites tumor cell TA3 treated with neuraminidase and then stained by the modified Hale method. The surface no longer reacts to give ferric ferrocyanide. × 15,000.

FIGURE 5 Section of a mouse ascites tumor cell TA3 used as a control for enzyme-treated cell in Fig. 4. This cell has a Hale-positive coat similar to that in Fig. 1. × 15,000.

FIGURE 6 Section of a mouse ascites tumor cell TA3. This cell was not exposed to colloidal iron during the staining procedure and so serves as a "blank." The absence of ferric ferrocyanide indicates that natural iron is not present to react with the potassium ferrocyanide. × 15,000.

FIGURE 7. Section of a mouse ascites tumor cell TA3 fixed with osmium tetroxide to identify the mitochondria. × 15,000.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is drastically altered. It is possible, of course, that this represents non-specific staining in the cytoplasm of the cell, or that it merely reflects incomplete exposure to the enzyme, or that these Hale-positive areas contain other types of acid mucopolysaccharides. This problem of internal staining specificity will be treated in future reports.

We believe the procedure is reasonably specific and hence valuable for cell surface studies and potentially valuable for internal structural studies when it is used with appropriate controls and when the results are interpreted with caution. For example, with this technique we are now investigating the relationships between the presence of sialomucins and both adhesiveness and stickiness (to be published). There is evidence that this may be an important factor in the problem of metastasis (2, 13, 14). It may also be possible with this technique to investigate the origin of these sialomucins and their transport within the cell. We intend further explorations along these lines.

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