Transformation-dependent Modifications in Released and Cell-bound Surface Proteins Detected by Antisera to Shed Antigens

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SUMMARY

Antiserum against antigens released into the medium by rat cell cultures that express the transformation phenotype has been shown to produce a complement-mediated lysis, not only in the cells that liberate such antigens but to a greater extent in cells that are phenotypically restricted in the expression of transformation properties.

Reaction of the antibody with preiodinated surface proteins released into the medium reveals that the expression of transformation leads to a greater degradation of the external proteins shed into the medium.

INTRODUCTION

One of the clearer modifications that appears to be associated with altered growth behavior of malignant cells is that which relates to a decrease in large, externally labeled glycoproteins detectable preferentially in untransformed cells (1–6).

Recent studies in mammalian cells (2, 6) and avian systems (3, 5) have revealed that an important reason for the decrease of such large external transformation-sensitive proteins appears to be due to an increased turnover and shedding of such component(s) (2, 6). Such results strongly suggest the presence of the large external transformation-sensitive protein or perhaps a processed form of it in the released medium (2, 6), in which proliferating, normal, and transformed cells are grown.

Although a recent report has shown that the external transformation-sensitive protein of chick embryo fibroblasts appears to be related to events involved in cell adhesion (7), little else is known about the biological role of such protein and, in particular, about its functional relationship to the shedding process.

To gather more information about the shedding process and cell surface modifications in mammalian cells, we have now investigated whether antisera against shed antigens can detect differences in rat cells that either permit or restrict the expression of transformation.

We have now observed that cells restricted in the expression of transformation consistently exhibit a greater degree of complement-dependent lysis upon exposure to such antisera.

Also, use of the same antisera against components of the culture medium from cells expressing the transformed and normal phenotypes reveals that, in the latter case, cells shed cell surface components of a higher molecular weight than those shed during the expression of transformation.

MATERIALS AND METHODS

Cultures. The cells used in this study were a clone (ts-NT3-KR) from an isolate of normal rat kidney cells (NRK) infected with the temperature-sensitive mutant of the B77 strain of Rous sarcoma virus. These cultures exhibited properties of transformed cells when grown at 33° and of the normal phenotype when seeded at 37°. At the lower temperature, the cells exhibited a typical morphology of transformed cells, revealing also an increased deoxyglucose uptake, formation of colonies in agar, greater agglutinability, and growth to a higher saturation density than at 37° (2). For the experiments to be described, cultures were seeded at a concentration of 2.5 × 10⁶ cells/cm² in Dulbecco’s medium (Grand Island Biological Co., Grand Island, N. Y.; Catalog No. H-16) supplemented with 10% fetal calf serum, 10% tryptose phosphate broth, and 1% dimethyl sulfoxide. Cell monolayers, which were about two-thirds confluent, were exposed to about 10 μCi ⁵¹Cr (NaCrO₄, Catalog No. CJZ-84, obtained from Amersham/Searle Corp., Arlington Heights, Ill.) for 90 min prior to exposure to the antisera, wherever indicated. The ⁵¹Cr experiments and those in which cell damage was assessed by trypan blue staining were preceded by careful medium change and 2 washes in fresh medium without serum to eliminate a blocking effect on the activity of the antisera by antigens possibly present in the culture medium.

Antigen Source. ts-NT3-KR cells were seeded as described above in medium supplemented with 0.5% serum. Following a 24-hr period at either 37 or 33°, cell monolayers were washed carefully 5 times in medium without serum and subsequently exposed for 36 hr to fresh medium without serum. Prior to use, the media were dialyzed against water extensively and subsequently lyophilized. Antisera were prepared from the lyophilized conditioned media by...
immunizing rabbits with 500 μg of material emulsified in Freund's complete adjuvant. The immunization schedule was repeated 3 times at 4-week intervals. Immunodiffusion analysis of the antisera prepared against antigens collected from cells grown at 37 or 33° revealed identity patterns. However, most of the antisera showed stronger precipitation lines with material prepared from cells grown at 33°. Thus, the experiments to be described were carried out with antisera against antigens shed at 33°.

Iodination and Electrophoresis. Surface labeling was carried out by the lactoperoxidase-catalyzed radioiodination (2). Subsequently, cells were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2). Gels were either stained by Coomassie blue (2) or sliced in 1-mm fractions, and radioactivity was eluted from the slices by extraction with 0.5 N NaOH at 80° for 1 hr and further for 16 hr at 37°. Subsequently, samples were cooled for 3 hr to avoid chemoluminescence and counted prior to the addition of Bray's fluid.

Immune Precipitation. Precipitation was carried out by reacting 25 μl of homologous nonimmune serum or immune serum with identical protein concentrations of conditioned media from cells grown at 37 or 33°. After incubation at 37° for 30 min and overnight incubation at 4°, immune precipitates were collected at 12,000 x g for 10 min, washed 3 times in 0.9% NaCl solution, and dissociated as described elsewhere for sodium dodecyl sulfate-gel electrophoresis (2).

Measurement of 51Cr Released. Measurement was carried out by taking duplicate aliquots at the various time intervals indicated. Samples were cooled prior to the addition of Bray's fluid and subsequently counted on a liquid scintillation spectrometer, with adjustments similar to those used for tritium measurements.

RESULTS

For the experiments to be described, NT3-KR cells grown at 37° exhibited a very flat morphology typical of untransformed cells, whereas parallel cultures seeded at 33° showed growth in patches and significant refractility typical of transformed cells. Incubation of the cells with antibody in the presence of complement did not lead to significant alterations in the morphology of cells grown at 33°, although an identical treatment did mediate a rounding effect on the otherwise flat cells grown at 37° after a 60-min incubation with the antiserum prepared against shed antigenic components. A most interesting effect was that the gradual rounding of the cells grown at 37° as a result of exposure to the antibody increased in proportion to the dose of antibody after the 60-min period. No comparable rounding was observed in the cells, merely exposed to complement or antibody alone, that were grown at 37°.

Examination of the viability of the cells exposed to antibody and complement by their ability to exclude trypan blue is described in Table 1.

It becomes apparent that, at every concentration of antibody used, there is clearly a greater complement-dependant damage to cells grown under conditions that restrict the expression of transformation. Although high doses of antibody were found to affect markedly both the cells expressing the untransformed and transformed phenotypes, nevertheless, at lower doses there is clearly an apparent differential effect. Parallel experiments in which cells were prelabeled for 90 min with 51Cr prior to exposure to antibody and complement are presented in Chart 1. This shows that, after a lag of 45 to 60 min, cells restricted in the expression of transformation begin to release a clearly greater amount of 51Cr, indicating that also by this criteria such cells exhibit a greater damage by the antiserum than the cells grown under permissive conditions for the expression of transformation.

The above results demonstrate that antisera prepared against antigens shed by proliferating cells can be used to monitor surface differences between cells that express untransformed and transformed differences.

That the above results are related to the expression of the transformation phenotype and do not merely represent a temperature effect is assumed because: (a) although ts-NT3-KR cells were grown at different temperatures, the assays with the antiserum and complement of Table 1 and Chart 1 were both carried out at 37°; (b) essentially similar findings were obtained when comparing the uninfected parent NRK cells with the transformed derivative infected by the wild-type transforming Prague virus, a variant of the B77 Rous sarcoma virus.

In such experiments, we observed that about 9 to 11% of infected "normal" cells took up trypan blue when exposed to 0.1 ml of antiserum against shed antigens and 0.1 ml of fresh normal rabbit serum, in comparison with only 2 to 3% of stained cells in the case of cultures of rat cells transformed by the Prague strain of Rous sarcoma virus.

Although immunodiffusion tests revealed that antisera against antigens shed from cells grown at 37 or 33° revealed identity patterns, we decided to test whether subsequent

Malignant Transformation and Shed Surface Antigens

Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Antiserum (ml)</th>
<th>Complement</th>
<th>% stained cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grown at 37°</td>
<td>0.1</td>
<td>Present</td>
<td>15</td>
</tr>
<tr>
<td>Grown at 33°</td>
<td>0.1</td>
<td>Present</td>
<td>1</td>
</tr>
<tr>
<td>Grown at 37°</td>
<td>0.2</td>
<td>Present</td>
<td>30–40</td>
</tr>
<tr>
<td>Grown at 33°</td>
<td>0.2</td>
<td>Present</td>
<td>5–8</td>
</tr>
<tr>
<td>Grown at 37°</td>
<td>0.4</td>
<td>Present</td>
<td>90–100</td>
</tr>
<tr>
<td>Grown at 33°</td>
<td>0.4</td>
<td>Present</td>
<td>30–45</td>
</tr>
<tr>
<td>Grown at 37°</td>
<td>None</td>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Grown at 33°</td>
<td>None</td>
<td>Absent</td>
<td>0</td>
</tr>
</tbody>
</table>

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fractionation of the immune precipitates by high-resolution polyacrylamide gel electrophoresis would reveal differences that could be correlated with the expression of transformation. For such purpose, the same antiserum was used in the experiments that described the complement-mediated lysis, including the corresponding controls with nonimmune serum. Use of control normal rabbit serum against the conditioned medium of proliferating cells is shown in Fig. 1. The corresponding polyacrylamide gel pattern obtained by reacting nonimmune serum with cultured media shows that a number of protein species are precipitated to a comparable extent both in conditioned medium from cells expressing the transformed phenotype at 33° and in that from cells restricted in the expression of transformation at 37° (Fig. 1).

Use of the immune serum prepared in rabbits (Fig. 2) against the antigens shed by the cells gives a precipitate which upon subsequent electrophoresis shows comparable gel patterns, although there is a trend toward the presence of a slower migrating component appearing more noticeable in the growth medium from cells grown at the temperature that restricts the expression of transformation and the presence of fast-migrating components in cells expressing the transformed phenotype (Figs. 1 and 2). Preabsorption of the culture media with nonimmune serum and use of the corresponding supernatants for precipitation with immune serum and subsequent gel electrophoresis are presented in Fig. 3. It can be seen clearly that there is a detectable difference in the species present in medium from cells grown at 37°, evidenced by the presence of a slow-migrating component with a molecular weight of about 180,000 to 200,000, which is clearly absent from the corresponding culture medium of cells grown at 33° (Fig. 3).

Although the above experiments show that cells expressing the transformed phenotype differ from cells restricted in the expression of transformation with regard to the protein species that are shed into the medium, no evidence is furnished from such experiments about the cell surface origin of such differences.

Based on the previous results of complement-mediated lysis (Table 1; Chart 1), which implied that the antiserum used was recognizing cell surface antigens, we labeled the exposed surface proteins from the cells by lactoperoxidase-mediated enzymatic radioiodination.

Fig. 4 shows a typical autoradiography of an electrophoretogram from cells grown at 37 and 33°, respectively. It can be seen that, whereas cells labeled at 37° show some radioactivity at the top of the gel in the molecular weight region of 250,000, as well as in 3 additional faster migrating regions, cells iodinated at 33° lack the high-molecular-weight component at the top of the gel (Fig. 4).

Such cells were iodinated and subsequently exposed to fresh complete medium with 10% serum to study the nature of the macromolecular 125I shed into the medium. One hr after the medium change following iodination, the corresponding conditioned media were allowed to react with immune serum against shed antigens. The corresponding immunoprecipitates were dissociated and assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Chart 2 shows that, whereas the medium from cells grown at 37° reveals a clear region of radioactivity in the molecular

![Chart 1: Transformation-dependent release of 125I by NT3-KR cells exposed to antiserum against shed antigens. Identical cell numbers were allowed to incorporate 125I and subsequently exposed for the time intervals indicated above to 0.1 ml of antiserum, and aliquots were collected for assay of radioactivity (see text). •, cells seeded at the restrictive temperature for the expression of transformation at 37°; x, cells seeded at the permissive temperature for the expression of transformation at 33°.](chart1)

![Chart 2: Electrophoretic pattern of reaction products by the antiserum to shed antigens with material released from lactoperoxidase-catalyzed radioiodinated NT3-KR cells labeled as indicated in Fig. 4. The products of immune precipitation were collected and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in (9-cm) 7.5% running gels and (1-cm) 3.3% stacking gels. Radioactivity was measured in 1-mm gel slices and processed as indicated in the text. ——, radioactivity released from cells grown at the restrictive temperature for the expression of transformation at 37°; ——, radioactivity released from cells grown at the permissive temperature for the expression of transformation at 33°.](chart2)
weight region of 200,000, no such comparable component is detectable in the medium from cells grown at 33°, when the transformation phenotype is preferentially expressed.

**DISCUSSION**

The fact that antisera prepared from antigens shed from transformed cells cause a complement-mediated lysis preferentially on cells restricted in the expression of transformation is compatible with the idea that similar antigenic determinants are exposed in the untransformed and transformed states. The greater lability or reactivity of untransformed cells to such antisera is consistent with a greater exposure of such surface antigens in the untransformed cell. The fact that the large external transformation-sensitive protein is 1 of the major alterations in cell surface components, during the expression of transformation, makes it tempting to think that such a component may be involved in the reaction reported above of a greater susceptibility in the untransformed cell. This latter assumption fits well with current data, in which untransformed cells exhibit a significantly lesser degree of turnover of their cell surface components than cells expressing the transformed phenotype (2).

As no clear information was hitherto available on the integrity of the surface components released by cells expressing the untransformed or transformed phenotypes, we have now made use of the antisera against shed antigens in an attempt to gather information on shed surface antigens. Because the data presented above suggested that the complement-mediated lysis caused by the antisera was recognizing surface antigens, we decided to prelabel the external surface of the cells with radioiodination to follow the macromolecular components released into the medium by immune precipitation with the antibody against shed antigens. Such experiments have revealed that, although the shed surface material from cells restricted in the expression of transformation appears mainly as a peak with an approximate molecular weight of about 200,000, the corresponding material is essentially absent from cells expressing the transformed phenotype. Such a result was obtained from cells that were enzymatically radioiodinated under identical conditions and essentially to the same extent. It then appears that the cells expressing the transformed phenotype are not just releasing cell surface components at a greater rate than the corresponding "normal cells" (2) but, in addition, the released surface components appear to be converted to a lower-molecular-weight form in the case of cells that express the transformed phenotype.

**REFERENCES**


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Fig. 1. Precipitates obtained from the reaction between conditioned media of NT-KR cells and normal rabbit serum. Left, reaction from material released by cells grown at 37°; right, reaction from material released by cells grown at 33°. The above profiles correspond to experiments carried out in 10% sodium dodecyl sulfate-polyacrylamide gel.

Fig. 2. Electrophoretic profiles of the precipitate obtained from the reaction between conditioned medium of NT-KR cells and serum against shed antigens prepared as described in "Materials and Methods." Left, material from cells grown at 35°; right, material from cells grown at 37°. The above profiles correspond to 10% sodium dodecyl sulfate-polyacrylamide gels.

Fig. 3. Electrophoretic profiles of the immune precipitation of the supernatant obtained from the preabsorption with normal rabbit serum of shed antigens released by NT-KR cells. Left, product obtained from the reaction of material shed by cells grown at 37°; right, product obtained from the reaction of material shed by cells grown at 33°. The above profiles correspond to the results obtained in 7.5% sodium dodecyl sulfate-polyacrylamide gels.

Fig. 4. Lactoperoxidase-mediated iodination radioactivity of NT-KR cells. Left, autoradiography of electrophoretograms from cells shed at 37°; right, autoradiography of electrophoretograms from cells shed at 33°. The above pattern corresponds to a 5% sodium dodecyl sulfate-polyacrylamide running gel. The upper component on the left corresponds to radioactivity that enters the stacking gel and appears at the beginning of the running gel.

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