Rabbit Antibodies to Nucleoli of Novikoff Hepatoma and Normal Liver of the Rat

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SUMMARY

Antinucleolar antisera were produced in rabbits immunized with whole isolated nucleoli from normal rat liver and rat Novikoff hepatoma ascites cells. These antisera produced positive nucleolar fluorescence of varying degrees in nucleoli and nuclei of Novikoff tumor, rat liver, kidney, and Walker tumor cells. Novikoff hepatoma and liver antinucleolar antisera fixed complement when combined with 0.15 M NaCl-soluble proteins extracted from the hepatoma and liver nucleoli. Nucleolar specificity of the antibodies was demonstrated by inhibition of fluorescence or complement-fixation following pretreatment of the immune sera with whole nucleoli or nucleolar 0.15 M NaCl-soluble protein fractions.

INTRODUCTION

Nucleoli of normal and tumor cells have been studied by a variety of methods (2). Light and electron microscopy have been used to detail the structures of the nucleoli of resting and growing cells. The development of methods for isolation of nucleoli from other cellular components (2) has led to a more refined biochemical analysis of nucleolar components (9). Among other methods, immunological procedures have also been used to study the internal structure of the nucleolus. For example, Ritchie (11) used a modification of the immunofluorescence technique for studies on nucleolar structure with sera of patients with antinucleolar antibodies.

Although antinucleolar antibodies are naturally occurring in a number of diseases, such as lupus erythematosus (1, 12), it was of interest to determine whether experimental antinucleolar antibodies could be induced as an aid to studies on nucleolar proteins from normal and neoplastic cells. In the present study rabbits were immunized with nucleoli from normal rat liver and Novikoff ascites tumor cells. The antisera produced exhibited nucleolar specificity as determined by immunofluorescence and CF.2

MATERIALS AND METHODS

Immunization Procedures. Nucleoli were isolated by the sucrose-Ca++ method (2) from normal liver and Novikoff ascites tumor cells from Sprague-Dawley or Holtzman male rats. The initial inoculation was divided; part was injected i.d. into the foot pads and part was injected i.m. into New Zealand white rabbits. Two additional i.m. inoculations were given at weekly intervals. The total amount of nucleolar antigen for each inoculation was 10, 20, and 50 mg, respectively, suspended in a total of 1 ml of complete Freund's adjuvant diluted 1:2 with 0.15 M NaCl. The blood was collected from the ear veins 7 to 10 days following the 3rd inoculation. Booster inoculations given at monthly intervals for 3 months consisted of 60 mg of tumor nucleolar antigen or 30 mg of normal liver nucleolar antigen suspended in incomplete Freund's adjuvant diluted 1:2 with 0.15 M NaCl.

Preparation of Nuclei for Immunofluorescence Studies. Nuclei were isolated from rat liver, kidney, Novikoff ascites tumor, and Walker tumor cells using the 0.5% citric acid method (13) as well as the 1.5% and 5% citric acid methods (2, 8). The isolated nuclei were suspended in 0.34 M sucrose, spread on glass slides, air dried for 18 to 20 hr at 4°, and fixed in acetone for 10 to 12 min.

Nucleolar Immunofluorescence. The procedure used for detecting immunofluorescence was modified from the indirect immunofluorescence technique of Hilgers et al. (6). The fixed specimens were overlaid with the antinucleolar antisera diluted 1:10 with 0.15 M NaCl. The slides were placed in a moist chamber and incubated for 40 min at 37°. The slides were washed 3 times in PBS for 5, 50, and 5 min and were air dried. Fluorescein-labeled caprine anti-rabbit IgG globulin diluted 1:5 (Hyland Laboratories, Los Angeles, Calif.) was added and the slides were incubated in a moist chamber at room temperature. The slides were rinsed, washed overnight in PBS at 4°, dried, and counterstained with Evans blue (0.06%). Coverslips were mounted using glycerol-PBS (1:1). After sealing, the slides were examined using a Zeiss fluorescence microscope (HB200, mercury bulb) with a BG12 excitation filter and a 530 nm secondary filter.

Absorption of Antinucleolar Antisera. Antisera were diluted 1:10 with 0.15 M NaCl, and approximately 45 mg of whole Novikoff tumor nucleoli or 45 mg of normal rat liver nucleoli were added to 1 ml of each diluted antisera. They were thoroughly mixed every 20 min during a 1-hr incubation at 37°. Nucleoli were removed by centrifugation for 30 min at 1700 X g and the antisera were tested for residual fluorescent antibodies with Novikoff tumor and liver nuclei.

CF Tests. CF was performed using microtiter techniques (10). Antinucleolar antisera diluted 1:10 with Veronal-buffered saline, pH 7.2, were inactivated at 56° for 30 min. Normal preimmunization sera served as controls. Serial 2-fold

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dilutions were made of the antisera at dilutions of 1:10 through 1:320 and of the 0.15 M NaCl-soluble antigenic proteins (100 μg/ml), and at dilutions of 1:2 through 1:64 with Veronal-buffered saline. Guinea pig complement, hemolysin, and sheep red blood cells were obtained from Baltimore Biological Laboratory, Baltimore, Md., and were standardized. A checkerboard type of titration with 2 exact units of guinea pig complement per well was carried out on plates at 4°C for 18 hr. After 0.015 ml of a 2.0% suspension of sensitized sheep cells was added to each well, the plates were incubated at 37°C for 1 hr.

For the CF tests the antigens were prepared by sonically dispersing whole nuclei in a 1.5% suspension in PBS for 5 min with a 1-min pause after each min of sonic dispersion followed by centrifugation at 16,000 × g for 30 min. After the protein content of the supernatant was determined (7), the protein concentration was adjusted to 100 μg/ml.

Inhibition of CF. The 0.15 M NaCl-solubilized liver or tumor protein (100 μg) combined with liver or tumor antinucleolar antiserum was kept at 4°C for 48 hr and then centrifuged at 1700 × g for 20 to 30 min to remove the precipitate. When excess antigen was found in preliminary CF tests, additional antiserum was added and the procedure was repeated. The final absorbed serum was then tested for CF antibodies. The amount of protein in the precipitate was determined in 0.1 N NaOH at 230 nm (4, 7).

RESULTS

Fluorescence of Nucleoli. Figs. 1 and 2 illustrate nucleolar fluorescence in various preparations. Table 1 summarizes the variations in the intensity of the nucleolar and nucleoplasmic staining. The nuclei of Novikoff hepatoma (Fig. 1, A and B), normal liver (Fig. 1, C and D), and Walker tumor (Fig. 2, A and B) were markedly fluorescent after incubation with the tumor antinucleolar antiserum and the fluorescein-labeled caprine globulin. By comparison, the kidney nuclei (Fig. 2C) were only very weakly fluorescent after exposure to the tumor antinucleolar antiserum, and in many nuclei the nucleoli were not fluorescent. The nucleoplasm was faintly visible in most of the nuclei after treatment with the tumor antinucleolar antiserum.

Table 1

<table>
<thead>
<tr>
<th>Nuclei source</th>
<th>Liver antinucleolar antiserum</th>
<th>Novikoff hepatoma antinucleolar antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleoli</td>
<td>Nucleoplasm</td>
</tr>
<tr>
<td>Novikoff</td>
<td>++++</td>
<td>–</td>
</tr>
<tr>
<td>Walker</td>
<td>++++</td>
<td>–</td>
</tr>
<tr>
<td>Liver</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>Kidney</td>
<td>±</td>
<td></td>
</tr>
</tbody>
</table>

Chart 1. Percentage of CF by antinucleolar antiserum. CF tests were carried out as described under "Materials and Methods." Antigens, 50 μg/ml, ±, tumor; o, liver.

Cross-absorption studies were carried out with both types of nuclei. Most of the fluorescence disappeared when tumor antinucleolar antisera were absorbed with liver nuclei and vice versa. However, the possibility that there are antigens in liver that are not present in the tumor was indicated by the trace fluorescence found in liver preparations after absorption of either antiserum with liver nuclei.

The nucleoli exposed to the liver antinucleolar antisera and the fluorescein-labeled caprine globulin, including the Novikoff hepatoma (Fig. 1, E and F), normal liver (Fig. 1, G and H), and Walker tumor (Fig. 2, D and E), were brightly fluorescent. The kidney nuclei (Fig. 2F) were somewhat less fluorescent than those of other tissues, but they were more fluorescent than were the kidney nuclei exposed to tumor antinucleolar antiserum. The nucleoplasm of Novikoff hepatoma and Walker tumor nuclei treated with liver antinucleolar antisera were less clearly defined than those treated with tumor antinucleolar antiserum (Fig. 1, E and F; Fig. 2, D and E). Nuclei treated with normal rabbit sera (Fig. 1, I and J) were not fluorescent.

Absorption of Antinucleolar Antiserum with Nucleoli. The tumor antinucleolar antiserum that was absorbed with whole Novikoff hepatoma nuclei did not produce nuclear fluorescence with Novikoff hepatoma nuclei; instead a greenish overall fluorescence was observed (Fig. 1K).

Absorption of the liver antinucleolar antiserum with whole liver nucleoli removed almost all of the fluorescent antibodies (Fig. 1L).3

CF Reaction. The results of the CF reactions with the antinucleolar antisera are shown in Chart 1. With tumor antinucleolar antisera the tumor nuclei antigen fixed complement at a higher serum dilution than did the liver nucleolar antigen. With the liver antinucleolar antisera there was a slightly higher titer for the liver antigen. The normal serum did not fix complement with either tumor or liver antigen.

Inhibition of CF. Table 2 shows that pretreatment of the liver antinucleolar antisera with liver or tumor 0.15 M NaCl-soluble proteins removed some antibodies. With 12-μg/ml amounts of either antigen, the amount of complement fixed decreased from 100% at a 1:80 dilution of antisera to 100% at a 1:20 dilution with the liver antinucleolar antisera fixed complement at a higher serum dilution than did the liver nucleolar antigen. With the liver antinucleolar antisera there was a slightly higher titer for the liver antigen. The normal serum did not fix complement with either tumor or liver antigen.

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3Cross-absorption studies were carried out with both types of nuclei. Most of the fluorescence disappeared when tumor antinucleolar antisera were absorbed with liver nuclei and vice versa. However, the possibility that there are antigens in liver that are not present in the tumor was indicated by the trace fluorescence found in liver preparations after absorption of either antiserum with liver nuclei.
The antiserum may be due to common antigenic proteins shared by antibodies reacted with 0.15 M NaCl-soluble nuclear proteins. It was demonstrated at least 2 types of nucleolar antigens. The CF tissues, i.e., the tumor antinucleolar antisera produced a strong specificity. They cross-reacted with nucleoli from the various tissues, i.e., the tumor antinucleolar antiserum produced a strong fluorescence with Novikoff hepatoma and Walker tumor but slightly less with normal liver and even less with kidney. Similarly, the liver antinucleolar antiserum produced a strong fluorescence with nucleoli of normal liver, Novikoff hepatoma, and Walker tumor. This result is not unexpected since the 2-dimensional gel method has shown that there are many common nucleolar proteins in the Novikoff hepatoma and normal liver (3, 9). The nucleolar fluorescence of liver nucleoli was greater with its specific antiserum than with tumor antinucleolar antiserum (Fig. 1E). It is not clear whether the difference reflects an "organ-specific antigen" that is lost during carcinogenesis (5).

Interestingly, the fluorescence of kidney nucleoli was also greater with the liver antinucleolar antiserum than with tumor antinucleolar antiserum. This result suggests there may be common proteins in liver and kidney that are present to a lesser degree in the tumor.

In addition, there appeared to be a degree of tissue specificity in the CF reaction. The liver antinucleolar antiserum produced more precipitate than did the tumor antinucleolar antiserum, as shown by the weight of the aggregate. In addition, there was a greater decrease in CF with the absorbed liver antinucleolar antiserum than there was with the absorbed tumor antinucleolar antiserum. The greater reactivity of the antibody to the liver nucleolar antigens may result from differences in components, particle sizes, or a greater

### DISCUSSION

The experimental objective of these studies was to determine whether whole nucleoli of rat tissues were capable of eliciting specific antibody formation in rabbits. Since nucleoli contain many proteins, it is not clear which or how many function as antigens. Although in diseases such as lupus erythematosus and Sjögren's syndrome various types of nuclear and nucleolar antibodies are produced (1, 12), the antibodies produced in this study exhibited nucleolar specificity. The nuclear fluorescence observed with the corresponding antibodies produced in this study exhibited nucleolar specific activity. The nuclear fluorescence observed with the corresponding antibodies produced in this study exhibited nucleolar specific activity.

### Protein-Antibody Precipitate

The combination of liver antinucleolar antisera with liver proteins produced the largest precipitate (Table 3). This same antiserum produced a smaller precipitate with the tumor proteins. With tumor antinucleolar antiserum, it was necessary to add additional antiserum to remove anticomplementary effects of excess antigen. Less precipitate formed in each tumor antiserum-protein combination than with the liver antinucleolar antiserum. However, the tumor antinucleolar antiserum-tumor protein combination produced a larger precipitate than did the tumor antinucleolar serum-liver protein combination.

### DISCUSSION

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The methods used for determination of antibodies demonstrated at least 2 types of nucleolar antigens. The CF antibodies reacted with 0.15 M NaCl-soluble nuclear proteins and the immunofluorescent antibodies detected nucleolar proteins that were insoluble in citric acid (2, 8).

The immunofluorescent antibodies are apparently nucleolus specific. They cross-reacted with nucleoli from the various tissues, i.e., the tumor antinucleolar antiserum produced a strong

### Table 2

**Percentage of CF by antinucleolar antisera pretreated with 0.15 M NaCl-soluble proteins**

The antisera were pretreated with either liver or tumor 0.15 M NaCl-soluble proteins as described under "Materials and Methods." The resultant treated antisera were tested for CF with tumor or liver antigen, 12 μg/ml.

<table>
<thead>
<tr>
<th>Antiserum-protein combination</th>
<th>Liver antinucleolar antiserum</th>
<th>Tumor antinucleolar antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:10</td>
<td>1:40</td>
</tr>
<tr>
<td>Liver antigen</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Tumor antigen</td>
<td>1:20</td>
<td>1:40</td>
</tr>
<tr>
<td>Antiserum pretreated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with liver proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver antigen</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Tumor antigen</td>
<td>1:20</td>
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</tr>
<tr>
<td>Tumor antigen</td>
<td>1:20</td>
<td>1:40</td>
</tr>
</tbody>
</table>

100% at 1:80 to 100% at 1:40 with the liver antigen and from 100% at 1:80 to 100% at 1:10 with the tumor antigen.

Table 2 also shows the comparative results of treatment of tumor antinucleolar antiserum with liver and tumor nucleolar 0.15 M NaCl-soluble proteins. A lesser decrease of the activity was noted, i.e., there was only a 1-dilution decrease for 100% activity with the tumor antigens and slightly less with the liver antigen in these studies.

### Table 3

**Quantitation of antigen-antibody precipitates formed with antinucleolar antisera**

The liver and tumor 0.15 M NaCl-soluble proteins, 100 μg/ml, were combined with liver antinucleolar antiserum 2 times, 0.2 ml, and 0.4 ml, or with tumor antinucleolar antiserum 3 times, 0.2 ml, 0.4 ml, and 0.6 ml. The precipitates were dissolved in 0.1 N NaOH and the amount of protein in the precipitate was determined at 230 nm.

<table>
<thead>
<tr>
<th>Antiserum-protein combination</th>
<th>µg precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver antinucleolar serum, 0.6 ml + liver proteins 100, µg</td>
<td>182</td>
</tr>
<tr>
<td>Liver antinucleolar serum, 0.6 ml + tumor proteins, 100 µg</td>
<td>145</td>
</tr>
<tr>
<td>Tumor antinucleolar serum, 1.2 ml + tumor proteins, 100 µg</td>
<td>100</td>
</tr>
<tr>
<td>Tumor antinucleolar serum, 1.2 ml + liver proteins, 100 µg</td>
<td>64</td>
</tr>
</tbody>
</table>
concentration of specific antigens in the liver. In this connection it is noteworthy that the fibrillar elements are in a much greater abundance in normal liver nucleoli than in the tumor nucleoli (2, 14).

It is the goal of future studies to isolate specific individual nucleolar proteins (9) and to use these methods to ascertain their immunogenic potentials (3) as well as their specific nucleolar localizations.

ACKNOWLEDGMENTS

We wish to thank Dr. Lynn Yeoman and Charles Taylor for the preparation of nuclei, and Dr. Matilda Benyesh-Melnick and Lewis J. Holley of the Department of Virology for help with the CF tests.

REFERENCES


Fig. 1. Photomicrographs of nuclei tested with antisera or normal sera by the indirect immunofluorescent technique. Tumor antinucleolar antisera were incubated with Novikoff hepatoma nuclei (4, × 1800; 6, × 4500) and liver nuclei (6, × 1800; 4, × 4500). Liver antinucleolar antisera were incubated with Novikoff hepatoma nuclei (4, × 1800; 6, × 4500) and liver nuclei (6, × 1800; 1, × 4500). Normal rabbit sera were incubated with Novikoff hepatoma nuclei (1, × 1800) and liver nuclei (6, × 1800). Tumor antinucleolar antiserum absorbed with Novikoff tumor nucleoli was incubated with Novikoff nuclei (K, × 1800), and liver antinucleolar antiserum absorbed with liver nucleoli was incubated with liver nuclei (L, × 1800).
Fig. 2. Photomicrographs of nuclei tested with antisera by the indirect immunofluorescent technique. Novikoff hepatoma antinucleolar antisera were incubated with Walker nuclei (A, × 1800; B, × 4500) and kidney nuclei (C, × 1800). Liver antinucleolar antisera were incubated with Walker nuclei (D, × 1800; E, × 4500) and kidney nuclei (F, × 1800).
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