Two-Dimensional Immuno-osmophoresis of Adenovirus Type 7 Antigens

S. K. VERNON AND B. A. RUBIN

Research and Development Department, Wyeth Laboratories, Inc., Philadelphia, Pennsylvania 19101

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A test is described in which adenovirus type 7 antigens are separated by two successive perpendicular applications of electrophoresis through agar, causing precipitin formation with cationic immune globulins. Three bands produced by adenovirus cultures distinguish among hexon, fiber, and penton antigens.

Studies on the morphology of adenovirus antigens have led to the identification of "hexon" and "penton" capsomers, the latter formed by a base with an attached fiber, as the structural subunits of the icosahedral capsid (4). The hexon is associated with group-specific complement-fixing (CF) activity. In preparations of adenovirus types 3, 4, 7, 9, and 11, a 12-pentonaggregate is responsible for "complete" hemagglutination (HA) of erythrocytes (8-11, 13; Stasny et al., submitted for publication); the type 5 penton hemagglutinates by itself (19). "Incomplete" HA, which requires the presence of heterotypic antiserum, is accomplished by single pentons and, with types 4 and 5, by fiber antigens (8-11, 19). An "HA-inhibition consumption" test is used to identify the fiber antigens of types 3, 7, 9, and 11 (8, 10-12; Vernon et al., submitted for publication).

Several immunodiffusion techniques have been used to assay for adenovirus antigens. Immunoelectrophoresis has detected three antigens of type 5 (2, 3). Immuno-osmophoresis (5, 16) has detected the same antigens with high sensitivity (6, 7).

In this laboratory no double-diffusion or electrophoretic test demonstrated more than two antigens in type 7 preparations. The two-dimensional test described in this report, combining the sensitivity of immuno-osmophoresis with the antigen separation obtained by immunoelectrophoresis, revealed a third type 7 antigen hitherto not clearly detectable. Cross-reacting antigens should be more easily detected by it than by one-dimensional immuno-osmophoresis.

MATERIALS AND METHODS

Adenovirus type 7 was grown on HeLa cell monolayers maintained on Eagle's basal medium without serum (Grand Island Biological Co., Grand Island, N. Y.). At the appearance of 90 to 100% cytopathogenic effect, the cells were collected by centrifugation and frozen and thawed five times in a small volume of 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 8.0. After being clarified by centrifugation, these harvested materials were subjected to either equilibrium isodensity centrifugation in cesium chloride or rate-zonal centrifugation in sucrose gradients.

To concentrate soluble antigens, the virus materials were adjusted to a specific gravity of 1.30 with solid CsCl and centrifuged for 72 hr at 35,000 rev/min in the SW-39 rotor of a Spinco model L ultracentrifuge. For rate-zonal centrifugation, 0.5 ml of virus material was layered onto a 4.3-ml gradient of 10 to 25% sucrose in phosphate-buffered saline. The gradients were then centrifuged for 2 hr under the above conditions.

Fractions were collected dropwise from the tube bottoms and assayed for CF (18), complete HA, and incomplete HA (9) activities with a Microtiter kit (Cooke Engineering Co., Alexandria, Va.). Rabbit antisera against adenovirus types 4 and 7 were obtained from Microbiological Associates, Inc., Bethesda, Md. The first (anti-type 4) was required for the detection of incomplete hemagglutinins. After suitable absorption (as described), the type 7 antiserum was employed directly in the electrophoresis studies.

For electrophoresis, baths containing a barbitone-acetate buffer (pH 8.6, 0.1; reference 14) from Colab Laboratories, Inc., were used. Microscope slides, 3.25 by 4 inches (8.25 by 10 cm), were covered with 10 ml of 0.6% Ionagar no. 2 (Colab Laboratories) dissolved in this buffer. Sample wells (6 mm diameter) were cut in the agar and filled with antigen preparations. A potential of 5 v/cm of agar path length, at a current of 12 to 14 ma per slide, was applied for 2 hr at 4°C.

Troughs (40 by 1 mm) were then cut parallel to the direction of the run and filled with rabbit antiaadenovirus type 7 serum. To remove nonviral-specific antibodies, this serum previously was absorbed by the agamma calf serum in which the HeLa cells had been grown. The equivalence ratios of calf serum to antiserum were determined by the method of Preer (15).

The resulting band pattern was transferred to a suitable medium for electrophoresis and subjected to a decision-making algorithm (17) for two-dimensional separation. After electrophoresis, the gel was fixed in formaldehyde-saline and stained for 12 hr with 0.01% Coomassie blue R-250 (pH 7.5) in 10% methanol and 5% acetic acid. The gel was then destained in 7.5% acetic acid and fixed in formaldehyde-saline for 12 hr. After electrophoresis, the gel was fixed in formaldehyde-saline and stained for 12 hr with 0.01% Coomassie blue R-250 (pH 7.5) in 10% methanol and 5% acetic acid. The gel was then destained in 7.5% acetic acid and fixed in formaldehyde-saline for 12 hr.
For immunoelectrophoresis, the slides were incubated at room temperature and antiserum was allowed to diffuse for 3 to 4 days. For two-dimensional immunooosmophoresis, the slides were so oriented as to undergo electrophoresis at right-angles to the first run, with the antiserum troughs nearer the anodic side. The same potential was again applied for 4 to 5 hr. The slides were then washed for 5 days in saline solution, dried, and stained with amido black. Double-diffusion tests were run on slides covered with 1% iodagar no. 2 dissolved in water or phosphate-buffered saline.

RESULTS

More than 90% of the CF and HA activities were found in the CsCl-banded fractions that had a specific gravity of 1.28 to 1.31. Table 1 shows the results of serological assays of fractions from rate-zonal centrifugation in sucrose gradients. Isopycnic-banded antigens and fractions 6, 9, and 11 from sucrose gradients were subjected to immuno-osmophoresis, and the resultant bands were numbered in order of appearance (Fig. 1). Band 1 was associated with group-specific CF activity and has been shown by electron microscopy (unpublished data) to be produced by type 7 hexons. Band 2 was associated with complete HA activity and was the only band formed by hemagglutinin partly purified by rate-zonal centrifugation. It was eliminated by treating the antigen with 0.1% trypsin at 37°C for 1 hr or by heating it for 30 min at 56°C. Band 3 probably was formed by the fiber antigen; the precipitin was found near the meniscus, well above the CF antigen peak, after sucrose gradient centrifugation. Parallel experiments showed that HA-inhibition-consuming antigen could be found in analogous fractions.

Immunoelectrophoresis of the same preparations and development with antiserum for 3 to 4 days resulted in the formation of only bands 1 and 2. Similarly, only two bands were visible after one-dimensional immunooosmophoresis. The failure of the latter test to detect the third precipitin may have been due to absorption of cross-reacting antibodies by the precipitin of band 2, which presumably contains the fiber antigen as well as penton bases.

DISCUSSION

Band 1 was produced by type 7 hexons, which migrated similarly to those of type 5 during agar electrophoresis (2, 3).

Band 2, although associated with complete HA activity, may have been formed by pentons arising from the breakdown of the complete antigen in the electric field. High incomplete HA: complete HA ratios, in rate-zonal purified hemagglutinin fractions, indicate the predominance of single pentons. Moreover, in right-angle diffusion tests (1, 2), the diffusion coefficient of the second precipitin was found to be 2.49 × 10⁻⁷ to 2.82 × 10⁻⁷ cm²/sec, close to the value of 2.6 × 10⁻⁷ cm²/sec obtained for type 5 pentons (2). During electrophoresis, the type 7 antigen (penton) migrated somewhat faster than that of type 5, compared to the respective hexons.

The third type 7 antigen migrated as a weak anion and was therefore detectable by the present method. This antigen was not demonstrable by one-dimensional immunooosmophoresis or by conventional immunoelectrophoresis. Although

TABLE 1. Adenovirus type 7 antigen content in the various sucrose gradient fractions subjected to two-dimensional immunooosmophoresis

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Complete HA</th>
<th>Incomplete HA</th>
<th>CF antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>128</td>
<td>1,024</td>
<td>≤4</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>8</td>
<td>1,024</td>
</tr>
<tr>
<td>11*</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>256</td>
</tr>
</tbody>
</table>

* Fractions from parallel runs contained HA-inhibition-consuming antigen at this position.

![Image](http://aem.asm.org)
the type 5 fiber is cationic (2, 7), the decreased endosmotic flow permitted by long agar no. 2 can account for the anionic position of this band.

The two-dimensional test has two advantages over previously reported methods. (i) It can differentiate between cross-reacting antigens migrating in one direction, and (ii) it is more sensitive and faster than conventional diffusion methods. Cationic antigens would presumably escape detection by this method, but the use of phenylsulphonated antiserum (17) should overcome this shortcoming without essentially altering the test.

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LITERATURE CITED