Recovery and Characterization of Non-cytopathogenic Rhinoviruses

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One of the first successful demonstrations of the presence of rhinoviruses in vitro was by the interference technique (Hitchcock & Tyrrell, 1960). The use of this technique became unnecessary following the subsequent finding that rhinoviruses readily produced c.p.e. in cell cultures if maintenance medium contained a low bicarbonate concentration and cultures were rolled at 33 °C (Tyrrell & Parsons, 1960). The development and use of human diploid cell strains (Hayflick & Moorehead, 1961) provided even more sensitive systems for the recovery of viruses of this group (Hamparian, Kettler & Hilleman, 1961).

Recent evidence, however, suggested that not all rhinoviruses are cytopathogenic even when cultivated under these ‘common cold’ conditions. Hoorn & Tyrrell (1966) described a strain of rhinovirus isolated in organ cultures of human embryo trachea which could not be adapted to grow in cultures of human diploid cells.

During studies of respiratory illnesses in military recruits, three virus strains were isolated in human embryonic kidney (HEK) cells by use of the interference technique. None produced c.p.e. before many passes in appropriate cell cultures although each possessed the properties of rhinoviruses. The results presented here suggest that the assay for interference offers a useful test for the efficient recovery of rhinoviruses.

Monolayer cultures of the cells specified below were maintained in tubes on a variety of media suitable for cultivation of rhinoviruses (Hamre, 1968). Media included Eagle’s basal medium with Earle’s salts (EBME) and 2 % foetal bovine serum (FBS); lactalbumin hydrolysate (LAH) with 5 % calf serum; L-15 and 2 % FBS; diploid maintenance medium (49 % Parker’s medium 199, 49 % EBME and 2 % FBS); or medium 199 with 2 % FBS. All media contained 0.03 % bicarbonate except one lot of EBME which employed tris buffer solution, pH 7.4. All sera used in maintenance media were heat-inactivated at 56 °C for 30 min before use.

Throat gargles were inoculated initially into tube cultures of WI-38, HEK and primary African green monkey kidney (GMK) cells. When no c.p.e. was observed after incubation at 33 °C or 37 °C for 14 days, approximately 1000 TCD50 (dose for infection of 50 % of culture) echovirus type 11 was added to 1 tube of each cell type, and to uninoculated control tubes. When the total controls showed degeneration following echovirus challenge, usually within 2 days when incubated at 37 °C, the presence of less than 25 % destruction of the test culture was taken as evidence of interference by virus in a primary sample.

Properties of virus strains. Titrations by the interference technique in monolayer cultures were made by inoculating four HEK culture tubes/dilution. These were incubated at 33 °C for 7 days. Activities in interference are expressed in terms of the highest dilution which caused interference in 2 of the four test cultures (lnD 50). Methods previously described were employed for assessment of chloroform sensititivity (Feldman & Wang, 1961), cation stabilization (Wallis & Melnick, 1962), acid lability (Ketler, Hamparian & Hilleman, 1962), provisional nucleic acid determination (Webb, Johnson & Mufson, 1964) and size (Hsiung, 1965). The rhinovirus serotypes used for comparison were obtained from Research Reference Reagents Branch, National Institute of Allergy and Infectious Diseases.

Isolation of strains. During the period of study 11 typical rhinovirus strains were isolated which readily produced c.p.e. In addition, three virus strains were isolated which did not
Table 1. Characterization of virus strain 215 and of rhinovirus type 15 (strain 1734)

| Virus and strain   | Chloroform sensitivity* | Stabilization by MgCl₂ to inactivation at 50 °C† | Log depression of infectivity at pH 3.0‡ | Sensitivity to IUDR§ (nm) | Size by filtration (nm) ||
|-------------------|-------------------------|---------------------------------------------|------------------------------------------|--------------------------|------------------|
| Agent 215         | -                       | -                                          | Over 3.3                                  | -                        | 10 to 50         |
| Rhinovirus type 15| -                       | +                                          | Over 2.3                                  | -                        | 10 to 50         |
| (1734)            |                         |                                             |                                          |                          |                  |
| Echo II           | Not done                | Not done                                   | 1.0                                      | -                        | Not done         |
| Herpes simplex    | Not done                | Not done                                   | Not done                                 | +                        | Not done         |

Tests according to conditions described by: * Feldman & Wang (1961), † Wallis & Melnick (1962), ‡ Ketler et al. (1962), § Webb et al. (1964), ‖ Hsiung (1965).

produce c.p.e. but were detectable only by the presence of interference. The latter were detected only in HEK cells and then only after incubation for at least 12 days. During the original isolation and 2 reisolation attempts, HEK and WI-38 cells maintained on each of the media listed above were used. Cultures were incubated in stationary racks both at 33 and 37 °C, and WI-38 cultures were also rolled at 33 °C. Cultures were consistently negative for c.p.e. after observation periods of 17 to 28 days, but were positive on each occasion for interference in HEK cells, after incubation at either temperature for up to 21 days.

The three isolates were passed repeatedly to fresh cultures of WI-38 or HEK cells maintained on a variety of media. Passage resulted in the eventual production of c.p.e. by all three virus strains, but only after 5 to 8 serial passes in HEK followed by 4 to 7 passes in WI-38 cultures. Prior to this the three isolates had been inoculated into a variety of cell cultures known to be susceptible to rhinoviruses and confirmed by simultaneous titrations of rhinoviruses types 1A (JH strain), 3 (FEB), 17 (33342) and 26 (127-I). The cell types tested included HeLa, KB and primary rhesus monkey kidney in addition to WI-38 and HEK. All cells were incubated at 33 °C in media with low (0.03%) bicarbonate concentration. Interference was demonstrated consistently within 5 days of inoculation in cultures of HEK cells, but no c.p.e. was produced in up to 14 days after inoculation into any type of cell culture.

Properties of isolates. For each of the three virus strains the total time which elapsed in culture between isolation and selection for c.p.e. was considerable. Eventual selection could not be predicted during early passages. Thus, the reliability of the assay of interference allowed early characterization and confirmation of whether the interfering agents belonged to the rhinovirus group. Representative results are shown in Table 1 for strain 215 and a well-characterized rhinovirus, type 15. All three strains showed the similar properties of a small, chloroform-resistant, acid-labile RNA virus of the rhinovirus group. Strain 215 differed from rhinovirus type 15 in that thermolability at 50 °C was not stabilized by cations, but this is an inconsistent characteristic of rhinoviruses (Dimmock & Tyrrell, 1962).

Reciprocal cross-neutralization tests suggested that strains 215 and 225 were indistinguishable, but distinct from strain 254. Because of the large number of serotypes present in the rhinovirus group, further antigenic studies were not made.

Mechanism of interference. The growth of strain 215 was determined by inoculating each of a large series of tubes of HEK cell cultures with five InD 50 of virus. Cultures were incubated at 33 °C and each day after inoculation six tubes were harvested by freezing and thawing three times. The pooled sample was clarified by centrifugation and 0.1 ml of the supernatant fluid assayed for virus content. The development of interference was followed...
by the daily challenge with echovirus type 11 of four tubes each of inoculated and uninoculated cultures. The presence of an interferon-like activity was identified in the fluids from infected cultures. Fluids were acidified to pH 3.0 with perchloric acid, stored for 18 h at 4 °C, centrifuged to remove debris, and the supernatant fluids restored to pH 7.0 with 10 N-NaOH. Dilutions (1.0 ml) were added to washed HEK and GMK cell cultures in tubes and allowed to adsorb for 24 h before the cultures were washed three times and inoculated with 32 TCD50 echovirus 11. The results (Fig. 1) show that resistance to challenge virus was first detected 2 days after inoculation and persisted thereafter. Acid-treated supernatant fluids contained interferon-like activity at the levels shown for HEK cells; this activity was first detected in supernatant fluids 6 days after virus inoculation and was absent when tested in GMK cells.

Selection by passage in human diploid cell cultures. Even though serial passage resulted in cytopathogenicity, the results of Table 2 suggest incomplete selection. All titrations were made in cultures of HEK cells using 4 tubes/dilution, and interference activities were determined after incubation for 7 days at 33 °C. Because of the much more rapid destruction caused by the challenging echovirus, interference was readily detected despite c.p.e. due to rhinovirus. Interference by either strain 215 or 225 was detectable to a much higher dilution than was the c.p.e. Although prototype rhinovirus strains also produced interference in cell cultures, they did so only at the dilutions at which c.p.e. was detectable.

These studies on three rhinovirus isolates indicate that these are non-cytopathogenic in cell cultures, but may be detected by interference in HEK cells. Although selection for cytopathogenicity was possible, this required at least 12 passages. By the use of an interference assay, the agents could be characterized immediately. The use of several cell types maintained on a variety of media, all suitable for cultivation of other rhinoviruses, ensured
Table 2. Comparative titrations using end-points in HEK cells for interference and cytopathogenicity

<table>
<thead>
<tr>
<th>Virus and strain</th>
<th>Log (InD 50/0.1 ml)</th>
<th>Log (TCD 50/0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>215 (low passage)</td>
<td>5.0</td>
<td>0.3</td>
</tr>
<tr>
<td>215 (high passage)</td>
<td>5.3</td>
<td>3.3</td>
</tr>
<tr>
<td>225 (low passage)</td>
<td>4.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Rhinovirus type 1 A (3H)</td>
<td>4.3</td>
<td>3.0</td>
</tr>
<tr>
<td>Rhinovirus type 17 (173342)</td>
<td>3.3</td>
<td>4.1</td>
</tr>
<tr>
<td>Rhinovirus type 26 (127-1)</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Rhinovirus type 3 (FEB)</td>
<td>4.1</td>
<td>4.3</td>
</tr>
</tbody>
</table>

optimal conditions for virus growth. No c.p.e. was produced despite the efficient replication of virus indicated by the activities obtained. This suggests that these agents are less fastidious than those reported by Hoorn & Tyrrell (1966), which replicated in monolayer cell cultures. The detection of an interferon-like activity in supernatant fluids suggests that an interferon may be the mediator of interference in this system; this activity was not characterized further.

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