Rhinovirus Replication in Suspension Cultures of HeLa Cells[1] (34407)

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(Introduced by H. G. Cramblett)

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Physicochemical and immunologic studies on the rhinovirus subgroup of picornaviruses (1) have been handicapped by difficulties in obtaining large amounts of purified virus. The highest infectivity titers ($10^{5.0}$–$10^{7.0}$/ml) consistently obtained have been from monolayer cultures of HeLa cells (2). Attempts to use suspended cell cultures to prepare material with higher infectivity titers have been limited. Rosenthal was unable to propagate rhinovirus type 2 in HEp 2 cell suspension cultures (3). Although Stott and Tyrrell were unsuccessful in attempts to propagate HeLa cells in suspension, their preliminary experiments indicated that rhinovirus type 2 multiplied in such "maintained" cultures (4). In our study, HeLa cells were grown in suspension culture and used for propagation of rhinoviruses. The use of the system for conducting precise studies of rhinovirus replication and for preparing large amounts of virus for experimental purposes is described.

Materials and Methods. Viruses. Rhinovirus type 14, strain 1059 (1, 5) was used in these studies; its propagation in monolayer cultures of HeLa cells has been reported previously (2). Plaque and infectivity assays were done as described by Conant *et al.* (6).

Cell cultures. The source of the HeLa cells and the materials and methods used for preparation of monolayer cultures have been described elsewhere (2). These HeLa cells have incorrectly been called M-HeLa and also have been referred to as Harvard HeLa by other workers (4, 7). For initiation of suspension cultures, cells were scraped off glass and suspended in Eagle's minimum essential medium (Joklik modified, Grand Island Biological Co., Grand Island, New York) containing 5% uninactivated fetal calf serum. The cells were maintained at a concentration of 3–5 $\times$ 10^5/ml. Incubation was at 37°. Under these conditions, cell concentration doubled in 24–36 hr.

Infection of suspension cultures. Cells were pelleted by low speed centrifugation and resuspended to a concentration of 5 $\times$ 10^6/ml. After addition of virus, cell–virus mixtures were incubated for 1 hr at 33° under constant agitation. Subsequently, the cells were adjusted with fresh medium to a concentration of 3–5 $\times$ 10^5/ml and incubation was continued at 33°.

Concentration of virus. For concentration, a modification of the method described by Green and Piña was used (8). Cells in suspension were pelleted by low speed centrifugation 12–15 hr after infection, resuspended in 0.01 *M* Tris buffer, pH 8.1, and frozen and thawed twice. The mixture was treated once with an equal volume of fluorocarbon 113, layered onto 5.0 ml of CsCl (av density 1.5 g/cm^3) and centrifuged 4 hr at 25,000 rpm in the SW 25.1 rotor of the Spinco model L ultracentrifuge. The clear supernatant was discarded and part of the clear CsCl cushion was removed from the bottom of the tube. The remaining opalescent solution containing the virus was collected and adjusted to a density of 1.40 g/cm^3 with solid CsCl. Centrifugation was repeated at 39,000 rpm for 24–30 hr using the SW-39 rotor. The virus band was collected and used for plaque as-

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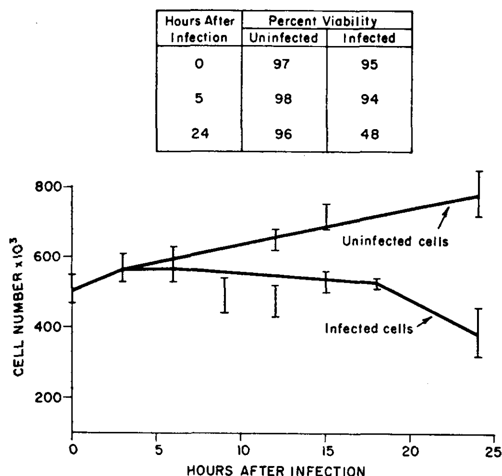

FIG. 1. Effect of rhinovirus infection on suspended cell cultures. The vertical bars represent high and low counts from six experiments.

says and determinations of buoyant density. The density (ρ_0) at 25° was determined using the formula:

$$\rho_0 = \frac{\text{net weight of sample}}{\text{net weight of H}_2\text{O}} \times 0.99707.$$

where 0.99707 is the density of H_2O at 25°.

Results. Effect of rhinovirus infection on suspended cell cultures. HeLa cell cultures were inoculated with multiplicities of 1 or 5 PFU/cell. Samples were taken for cell counts at various intervals. Cell counts were done in duplicate using a Neubauer ruled counting chamber. Cell viability was determined by staining with an equal volume of 0.2% trypan blue. Uninfected control cultures were handled similarly. Figure 1 shows the effect of rhinovirus infection on cell division and viability. In the infected cultures, no drastic change in total cell number was discernible until about 18 hr after infection. At this time there was a sharp decrease in total cell number, probably due to cell lysis. The number of viable cells dropped significantly 24 hr after infection. In contrast, uninfected cells increased 1.5- to 2-fold and retained viability.

Kinetics of adsorption. Cell cultures were inoculated with various multiplicities of infection. Samples were taken at intervals, clarified by centrifugation and supernatant fluids assayed for residual infectivity. Figure 2 shows that maximum adsorption occurred within 30 min regardless of input multiplicity. Approximately 40% of the infectious virus was adsorbed. Virus incubated for 2 hr in the absence of cells did not lose infectivity.

One-step growth curve. At intervals, samples were taken from infected suspension cultures and clarified by centrifugation. The supernates were used for assaying extracellular virus. The cells were washed twice with Earle's balanced salt solution, pH 7.4, and brought back to original concentration. Following two cycles of freezing and thawing, the clarified fluids were used to assay intracellular virus.

Nine hr after infection, there was a significant increase in intracellular virus with all input multiplicities (Fig. 3). Maximum yields of intracellular virus were obtained about 12 hr postinfection. Comparable levels of extracellular virus were not obtained until 30 hr.

Concentration of virus. Since the majority of virus produced by 12 hr was cell associated, this system was utilized to obtain high yields of infectious virus. The recovery of infectious virus at each stage in the concentration procedure is shown in Table I. Final recovery of viral infectivity was about 70%, reflecting a reduction in medium volume of

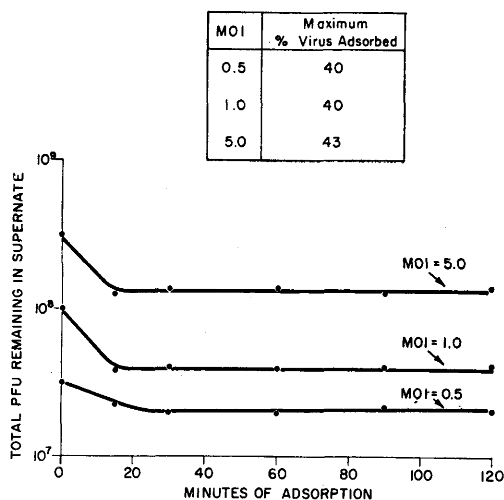

FIG. 2. Kinetics of rhinovirus adsorption with different multiplicities of infection (MOI).

TABLE I. Recovery of Rhinovirus Infectivity during the Course of Concentration.

Sample	(PFU/ml)	Total PFU	Total PFU (% of infectivity)
Cell pellet	5×10^7	1×10^9	100
Supernatant following Freon treatment of pellet	5×10^7	1×10^9	100
25,000 rpm			
Supernate	5×10^6	1×10^8	10
Cushion	1.8×10^8	9.0×10^8	90
Final virus band	6.8×10^8	6.8×10^8	68

about 1000-fold. In other experiments, the recovery of viral infectivity ranged between 50–80%.

The distribution of rhinovirus infectivity in fractions from CsCl gradients is shown in Fig. 4. The peak of infectious virus occurred at density 1.40 g/cm^3. Similar results have been reported by others (9–11).

Discussion. This study has shown that a member of the rhinovirus subgroup of picornaviruses replicates in suspension cultures of HeLa cells and that high yields of infectious virus can be achieved. In addition to results reported above, we also have obtained infectivity titers of 9.0 $\log_{10}$ TCD_{50}/ml with rhinovirus types 1A and 1B.

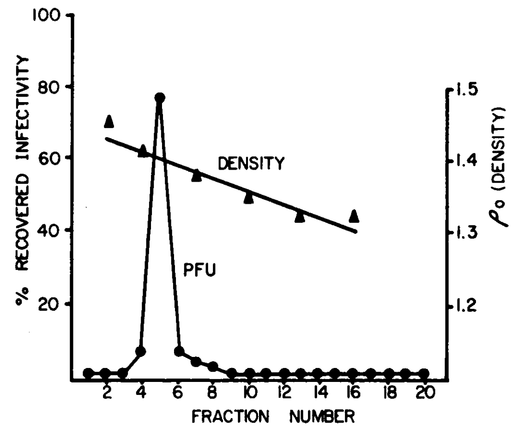

FIG. 4. Distribution of rhinovirus infectivity in fractions of CsCl gradients.

MOI	YIELD (PFU/CELL)
0.5	7.9
1.0	8.6
5.0	9.6

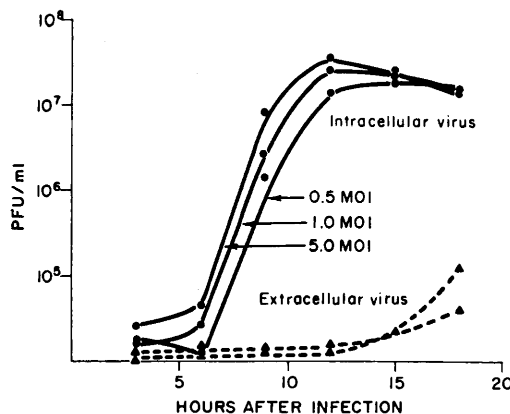

FIG. 3. Effect of multiplicity of infection on growth of rhinovirus in suspended cell culture.

In suspended cell cultures, the kinetics of adsorption was not affected by different input multiplicities and the 40% loss of infectivity observed within 30 min is consistent with values obtained in adsorption studies on HeLa cell monolayer cultures (6). However in the monolayer system, a 2-hr period was required to obtain maximum adsorption. These differences may have been due to the viral input employed. In the suspended cell system the input multiplicity was 0.5–5 while in monolayer cultures it was about 0.0004.

As has been the case with other viruses, the availability of a suspended cell culture system for propagation of rhinoviruses should allow quantitative biochemical studies to be performed with relative ease. Furthermore, the system should be useful for preparing large amounts of rhinovirus for physicochemical and antigenic characterization.

Summary. Rhinovirus-infected HeLa suspension cultures did not increase in cell num-

ber and lost viability late in the infectious cycle. Maximum adsorption occurred within 30 min with approximately 40% of the virus being adsorbed. Yields of intracellular virus of about 8–10 PFU/cell were obtained at 12 hr after infection. Comparable levels of extracellular virus were not obtained until 30 hr after infection. The suspended cell culture system should have practical value in studies on replication and for obtaining concentrated preparations of virions.

1. Collaborative Report on Rhinoviruses, Nature **213**, 761 (1967).

2. Conant, R. M. and Hamparian, V. V., J. Immunol. **100**, 107 (1968).

3. Rosenthal, M. S., J. Gen. Microbiol. **38**, 409 (1965).

4. Stott, E. J. and Tyrrell, D. A. J., Arch. Ges. Virusforsch. **23**, 236 (1968).

5. Conant, R. M. and Hamparian, V. V., J. Immunol. **100**, 114 (1968).

6. Conant, R. M., Somerson, N. L., and Hamparian, V. V., Proc. Soc. Exptl. Biol. Med. **128**, 51 (1968).

7. Manchee, R. J. and Taylor-Robinson, D., Brit. J. Exptl. Pathol. **50**, 66 (1969).

8. Green, M. and Piña M., Virology **20**, 199 (1963).

9. Dans, P. E., Forsyth, B. R., and Chanock, R. M., J. Bacteriol. **91**, 1605 (1966).

10. McGregor, S., Phillips, C. A., and Mayor, H. D., Proc. Soc. Exptl. Biol. Med. **122**, 118 (1966).

11. Gerin, J. L., Richter, W. R., Fenters, J. D., and Holper, J. C., J. Virol. **2**, 937 (1968).

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