JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1977, p. 202-207

Vol. 5, No. 2
Printed in U.S.A.

Demonstration of Dual Rhinovirus Infection in Humans by Isolation of Different Serotypes in Human Heteroploid (HeLa) and Human Diploid Fibroblast Cell Cultures

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Received for publication 9 September 1976

The ability to isolate rhinoviruses in human heteroploid cell cultures was investigated by inoculating HeLa cells (HeLa M) with specimens previously shown to be positive in human diploid cell cultures. The 135 positive specimens selected were representative of 22 different rhinovirus types, and 4 to 9 specimens were available for each serotype. Specimens were inoculated into human diploid fetal tonsil fibroblasts (FT), HeLa cells with 30 mM Mg^{2+}, and HeLa cells without increased Mg^{2+}. One hundred twelve rhinovirus strains (83%) were reisolated in FT cells, whereas 76 rhinovirus strains (56%) were recovered in HeLa cells with 30 mM Mg^{2+}. All strains recovered in FT were the same serotype as that originally recovered in diploid cells, but five of the HeLa cell isolates (3.7% of total specimens) were different serotypes, indicating dual rhinovirus infections. Four rhinovirus serotypes, (3, 42, 48, and 70) were recovered in HeLa but not in diploid cells; these serotypes were rare in our previous studies. Isolation of rhinovirus in FT cells was usually accomplished at first passage, whereas rhinovirus cytopathic effects in HeLa cells were not observed at first passage, but required one, two, or (rarely) three blind passages. Only 28 rhinoviruses (21%) were recovered in HeLa cells without increased Mg^{2+}; however, three serotypes, types 16, 36, and 58, were recovered as effectively in HeLa cells, with or without added Mg^{2+}, as they were in FT cells. In general, rhinoviruses were less efficiently recovered in HeLa cells; however, certain serotypes may be detected better by HeLa cells.

Specific etiological diagnosis of rhinovirus infections is needed for epidemiological studies, as well as for evaluation of possible control measures that might be available in the future (6, 8, 9, 18). Because of the large number of rhinovirus serotypes, serological surveillance is not feasible. Hence, we are interested in developing better and faster methods for rhinovirus isolation and identification. HeLa cells have been used successfully for all phases of rhinovirus studies (1, 2, 5, 16, 19) except direct isolation from specimens. This latter failure is puzzling, since rhinoviruses isolated in diploid cell cultures propagate in HeLa cells with 30 mM Mg^{2+} (5) to higher titers than in diploid cells. A recent report by Strizova et al. (17) of successful use of HeLa cells for rhinovirus isolation led us to conduct a direct comparison of a human diploid fetal tonsil (FT) cell line and HeLa M cell cultures for reisolation of rhinoviruses from 135 known virus-positive specimens. The FT cells yielded a higher proportion of isolates (84%) than HeLa cells, even with blind passage (56%). However, five specimens yielded two rhinovirus serotypes, one recovered in FT cells and the other in HeLa.

MATERIALS AND METHODS

Methods for rhinovirus isolation and typing have been described elsewhere in detail (3). Briefly, methods employed and materials used were as follows.

Specimens. All nasopharyngeal specimens studied had been stored at −70°C in a mechanical freezer. Specimens selected were known to be positive for a serotyped rhinovirus, and four to nine specimens were available per serotype. These included: 111 specimens (from 75 individuals) from the 1965 to 1969 Seattle Virus Watch program (7) that had yielded serotyped rhinoviruses in WI-38 cell cultures, 24 specimens (from 24 individuals) collected from ill children in 1974 and 1975 that had yielded serotyped rhinoviruses in FT cell cultures and, as controls, 100 specimens (from 100 individuals) from the 1974 to 1975 collection that had been screened in FT cells but yielded no isolates.

Cell cultures. The FT cell line used was a diploid

human fibroblastic cell line established in our laboratory by Saul Grinstein (7). HeLa M is a rhinovirus-sensitive cell line, originally obtained from the Merck Institute, which has been maintained in continuous culture for most of the past 10 years. HeLa 229 cells (a rhinovirus-insensitive strain) were obtained from San Pin Wang and are serial descendants (10 years' passage) of the HeLa 229 originally characterized for rhinovirus insensitivity (5), but sensitivity to chlamydiae (4, 10). The cell lines were tested by culture (11) and uracil incorporation (G. E. Kenny, *in* D. Schlessinger (ed.), *Microbiology–1975*, p. 32–36, American Society for Microbiology, Washington, D. C., 1975) regularly and were free of mycoplasmata. Growth medium for cells grown in monolayer was Eagle minimum essential medium (MEM) with 10% fetal bovine serum, and for HeLa M grown in suspension culture, Spinner MEM (Flow Laboratories, Rockville, Md.) with 10% fetal bovine serum was used. For virus isolation, medium for diploid cells was Leibowitz medium (GIBCO, Grand Island, N.Y.) with 2% fetal bovine serum. Medium for HeLa cells was MEM with 1% fetal bovine serum with or without 30 mM $MgCl_2$ as specified. Overlay medium for plaque assay in HeLa M consisted of MEM with 1% fetal bovine serum plus 30 mM Mg^{2+} containing 0.3% agarose (2, 5). Tube cultures (16 by 125 mm, screw capped) of heteroploid cells were prepared by inoculating 160,000 cells (from suspension culture for HeLa M) in 1 ml of growth medium and incubating at 37°C for 24 h before inoculation with specimens. Tube cultures of diploid cells were prepared using 150,000 cells and incubating at 37°C for 2 to 4 days until a monolayer was observed.

Rhinovirus isolation. FT cultures (two per specimen) were inoculated and incubated on a roller drum at 33°C for 14 days with examination for cytopathic effects (CPE) every other day. HeLa M tubes (two per specimen) were incubated similarly for 2 days after inoculation but, since cells degenerated spontaneously under these conditions, medium was changed and incubation was continued at 33°C in stationary racks for the last 5 days of the observation period. HeLa cell culture fluid was harvested at 7 days and blind-passed through two further passages. If CPE appeared, cell culture fluid was harvested and the agent was identified by neutralization.

Rhinovirus typing. Isolates were typed by neutralization, using monospecific rabbit antisera prepared in our laboratories (2), singly or, as indicated, in combined pools (12). Isolates that were not neutralized were tested for sensitivity to pH 3.

RESULTS

Isolation of rhinovirus in HeLa cells. Isolation of rhinoviruses from 135 specimens previously known to be positive for rhinoviruses (as tested in diploid cells) was attempted in HeLa M cells and FT cells (Table 1). A total of 112 rhinovirus strains (83.5%) were directly reisolated in FT cultures, whereas only 71 isolates (52.6%) were recovered in HeLa M cultures, after up to 4 blind passages with Mg^{2+}, and 28 isolates (21%) were recovered without Mg^{2+}. Four specimens yielded rhinoviruses in HeLa cells (the same type as originally isolated in diploid), but reisolation failed in FT cells. Seven rhinovirus serotypes, types 16, 19, 28, 36, 41, 58, and 68, were isolated as often in HeLa with Mg^{2+} as in FT. Three of these, types 16, 36, and 58, also were isolated efficiently in HeLa without Mg^{2+}. In contrast, six serotypes were isolated less effectively (types 1B, 22, 24, and 53) or not at all (types 65, 78) in HeLa cells, even with Mg^{2+}.

Effect of passage levels of diploid cells on isolation. The FT cell cultures employed for reisolation had had 21 to 28 passages. Since reisolations were somewhat less frequent in FT cultures at higher passage levels, 30 specimens negative for reisolation at the first inoculation were inoculated again into FT cultures that had had 17 passages. Although 11 rhinoviruses were reisolated from the 30 specimens, 7 of these caused CPE in only one of two tubes inoculated. This suggested that low virus content of specimens, rather than passage level of FT cells, was the critical factor in virus recovery.

TABLE 1. *Rhinoviruses reisolated in FT and isolated in HeLa M with and without additional Mg^{2+} in medium*

RV type previously isolated	No. of specimens tested	No. of specimens yielding expected rhinovirus type		
		In FT cells	In HeLa with MEM-Mg^{2+}	In HeLa with MEM-1[a]
1B	7	5	2	
8	4	4	3	1
10	5	5	3	
12	6	5	3	2
15	4	4	3	
16	8	6	7	7
19	8	6	6	2
22	7	6	2	
24	5	4	1	
28	7	4	4	2
36	6	5	5	4
38	6	6	3	
41	9	4	4	
53	5	4	1	
56	6	6	4	1
58	5	5	4	4
59	7	6	4	2
63	9	6	4	
65	4	4	0	
68	4	4	4	1
78	6	6	0	
81	7	7	4	2

[a] MEM-1, MEM plus 1% fetal bovine serum.

CPE. Rhinovirus CPE were first seen before day 5 after inoculation in FT cells in 22% of the 112 reisolations, and on day 5 or 6 in 62.5% (Fig. 1). Recognizable rhinovirus CPE were not seen in inoculated HeLa cells in the first passage. Almost all of the specimens positive in HeLa with Mg^{2+} showed CPE in the second or third passage. Only a few of the specimens were "suspicious" in the third passage and did not show typical rhinovirus CPE until the fourth passage. A more detailed analysis was carried out with the three types (16, 36, and 58) that were recovered with the same frequency in: FT, HeLa with 30 mM Mg^{2+}, and HeLa without Mg^{2+} (Table 1). In HeLa cells and 30 mM Mg^{2+} in the medium, 14 of 16 strains of types 16, 36, and 58 produced CPE at the second-passage level, whereas only 21 of 55 specimens, representing 17 other rhinovirus serotypes isolated in HeLa, produced CPE at the second passage level (Table 2). The effect of increased Mg^{2+} is also clearly demonstrated. Although the 16 isolates of types 16, 36, and 58 were recovered in HeLa cells without increased Mg^{2+}, only 3 strains produced CPE in second passage, whereas 7 and 6 were detected only at third and fourth passages, respectively. Of the remaining serotypes, only 22% were recovered, and 50% of these did not show CPE until the fourth passage.

Rhinovirus replication could be demonstrated by plaque assay in HeLa M at passage levels below those at which CPE could be detected. To examine the relationship of plaque production and CPE, five specimens, each containing a different rhinovirus serotype, were inoculated into HeLa M cells and passed through three successive passages on day 7 of incubation, or when cells showed 4+ CPE, and the remainder of the harvest was frozen at −70°C. Each successive passage was plated simultaneously on the HeLa cell monolayers for plaque assay. Plaque titers of 10^3 plaque-forming units per ml with type 16 and 10^5 plaque-forming units per ml with type 41 were seen in the passage level before CPE were detected (Table 3). Rhinovirus 1B and 68 produced plaques in the first-passage level, although CPE were not seen until the third-passage level. Type 22, which replicated poorly in HeLa cells, produced plaques at the third-passage level, at which time CPE were seen also.

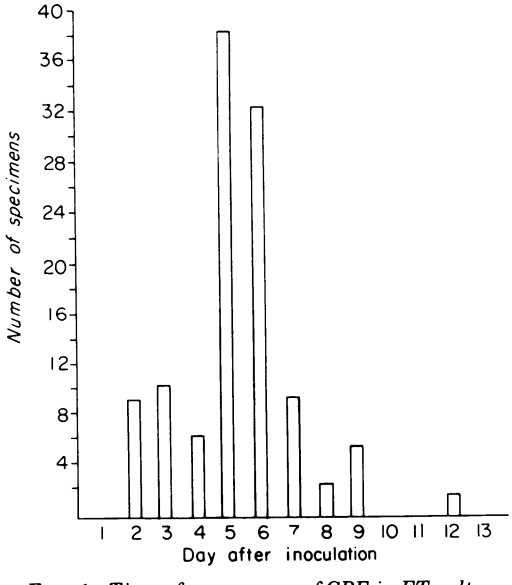

FIG. 1. *Time of appearance of CPE in FT cultures inoculated with specimens known to contain rhinoviruses.*

TABLE 2. *Number of passages required for isolation of rhinoviruses in HeLa M cells*

Rhinovirus types isolated	Passage level at which CPE first observed in HeLa cells							
	With 30 mM Mg^{2+}				Without added Mg^{++}			
	1	2	3	4	1	2	3	4
Types 16, 36, and 58	0	14	2	0	0	3	7	5
Other rhinovirus serotypes (17)	0	21	29	5	0	2	4	6

Dual rhinovirus infections. HeLa isolates that were not neutralized by antiserum to the rhinovirus type previously isolated were tested for acid sensitivity and identified by neutralization. As would be expected, some specimens yielded adenoviruses in HeLa that were not demonstrated in diploid cells. Three rhinovirus isolates, two type 19 and one type 81, were present in the HeLa with Mg^{2+} fluids, which also contained adenovirus type 2 isolates. The most surprising finding, shown in Table 4, was the recovery from five specimens of a second rhinovirus type in HeLa M, whereas FT cells yielded the serotype first recovered. The five strains were: two isolates of type 3, one isolate of type 48, and two isolates of type 42 (from specimens from one person taken 7 days apart). All typings were verified, and only one rhinovirus type was present in either FT or HeLa M cell culture fluids. These five isolates represent 3.7% of the total specimens examined.

Controls. In the second phase of the study, 100 rhinovirus-negative specimens collected during the same period as the rhinovirus-posi-

TABLE 3. *Development of CPE and plaque titer of rhinoviruses at successive passages in HeLa M cells*

Rhinovirus (RV) type in specimen	HeLa passage level							
	1[a]		2		3		4	
	CPE	Plaque[b] titer	CPE	Plaque titer	CPE	Plaque titer	CPE	Plaque titer
RV 16	0	1.5×10^3	4+, day 2	1.4×10^5	4+, day 1	4.3×10^6	4+, day 1	4.2×10^6
RV 41	0	No plaques	0	2.0×10^5	2+, day 3	2.1×10^6	4+, day 2	2.3×10^6
RV 68	0	5.0×10^4	0	3.6×10^5	1+, day 6	3.0×10^6	4+, day 2	3.3×10^6
RV 1B	0	1.5×10^2	0	Plaque assay not done	1+, day 6	3.0×10^4	4+, day 2	6.5×10^5
RV 22	0	No plaques	0	No plaques	1+, day 6	3.0×10^5	4+, day 2	3.4×10^6

[a] HeLa cell culture fluid harvested 7 days after inoculation with specimen.
[b] Plaque titer = plaque-forming units per milliliter.

tive specimens were inoculated into FT and HeLa M with Mg^{2+}. One CPE-positive, acid-labile, presumed rhinovirus (untypable) was isolated in FT cells. Two specimens yielded rhinoviruses, types 3 and 70, in HeLa M. A rhinovirus-insensitive HeLa line, HeLa 229, did not show CPE with specimens known to contain rhinovirus, or rhinovirus-infected FT or HeLa M cell fluids, which were inoculated and blind-passed in two further passages.

DISCUSSION

The data presented in this paper indicate that rhinovirus isolation is less effective in HeLa cells than in sensitive diploid cells; however, the sample is biased because the specimens selected were known to be previously positive in diploid fibroblasts. The reisolation rate from previously positive specimens was 56% in HeLa compared with 83% in our FT cell strain. The lower isolation rate in HeLa cells may be explained in part by our failure to maintain the cells longer than 7 days (cells could not be held longer because the monolayer deteriorated and CPE could not be observed nor could HeLa cells be rolled continuously), whereas the majority of the positive specimens did not produce recognizable CPE in FT cells until day 5 or 6 after inoculation (Fig. 1). However, HeLa cell culture fluids harvested on day 7 after inoculation produced plaques in HeLa cells under agar one or two passages earlier than CPE were seen (Table 3), suggesting that under the conditions of these experiments the limiting factor was our ability to observe CPE in HeLa cell cultures.

Perhaps the most interesting finding in these studies was the discovery of dual rhinovirus infections. Dual infections with other viruses occurred with some frequency in the Seattle Virus Watch (3), and many of these were identified by isolating one virus in one cell system and the second in another. Presumably, differential susceptibility of cell cultures for different viruses determines selection of one virus, if two are present. This phenomenon is apparently operative in FT and HeLa cells, since only one rhinovirus serotype appeared in each type of culture. It is probable that mixtures of two types could also occur in diploid cells, but such mixtures would be difficult to resolve and might explain some proportion of "untypable" isolates. The fact of dual rhinovirus isolates is intriguing in that it suggests a mechanism for the continued evolution of new types. Rhinoviruses replicating concurrently might offer opportunity for exchange of genetic information, resulting in recombinant progeny not typable by antiserum to either of the parent types. Extensive cross-reactions could also be explained by such a mechanism. Genetic recombination between different serotypes of foot-and-mouth disease virus has been shown (14), and it therefore seems reasonable to believe this could oc-

TABLE 4. *Different rhinovirus serotypes isolated from the same specimen in diploid cells and HeLa M cells*

Specimen	Serotype isolated in diploid cells	Serotype isolated in HeLa cells
1	RV 10[a]	RV 48
2[b]	RV 59	RV 42
3[b]	RV 59	RV 42
4	RV 8	RV 3
5	RV 1B	RV 3

[a] RV, Rhinovirus.
[b] Specimens collected from same person 7 days apart.

cur among rhinoviruses. If the dual infection rate found in this study (3.2% of 135 specimens) is indicative of true rates of dual infection in the population, recombination events might have a substantial opportunity to occur. The actual prevalence of mixed infections may be considerably higher, since the specimens selected for positivity in diploid cells were known to yield typable isolates (i.e., not mixed cultures). Unfortunately, sera were no longer available for testing for neutralizing antibodies to the infecting rhinovirus type isolated in HeLa cells.

An additional finding in these studies is the suggestion that certain rhinovirus serotypes may actually be isolated more easily in HeLa cells than in diploid cells. None of the virus types isolated only in HeLa cells (three isolates of type 3, two isolates of type 42, and one isolate each of types 48 and 70) happened to have been included in the study because we did not have sufficient specimens known to be positive for such types. Isolation of these types has been infrequent in diploid cells in the previous study of this population (7). Although the numbers are small, it is possible that these types are not effectively isolated in diploid cells but may require HeLa cells; studies to investigate this possibility are in progress. It should be noted that the experiments in the present study were biased against the detection of strains with HeLa tropism because of the choice of specimens previously known to be positive in diploid cells. Additional evidence of variability of susceptibility of cell lines to various rhinovirus serotypes was evident in this study. Thirty-four of 47 (72%) specimens containing seven rhinovirus serotypes, types 16, 19, 28, 36, 41, 58, and 68, yielded these viruses in both FT and HeLa cells. Inside of this cluster of viruses, types 16, 36, and 58 showed CPE relatively early in HeLa cells and increased Mg^{2+} was not required for their isolation. On the other hand, for the remaining 88 specimens, representing 15 serotypes, 42% were positive in HeLa and 89% were positive in FT cells (Tables 1 and 2). Thus, these data suggest that certain serotypes vary in their isolation efficiency in HeLa and diploid cells. The original division of rhinoviruses into "H" and "M" strains also recognized biological differences in specific serotypes (9).

The present data and conclusions need to be compared with the results of previous studies. Strizova et al. (17) reported that higher rhinovirus isolation rates were obtained in HeLa cells, as compared with diploid human embryonic lung cells when specimens obtained from volunteers infected with rhinoviruses were tested. These results are not directly comparable to the present studies, because the virus strains used to infect the volunteers had been passed in cell cultures. Some of the strains tested were isolated directly from the volunteers, and others had had at least one passage in diploid cells before parallel inoculation into HeLa cells and diploid cell cultures. In spite of the interspersing passages in volunteers with passages in cell culture, it seems likely that these rhinovirus strains were more like laboratory-passaged strains than isolates from field specimens. Six rhinovirus serotypes were studied: 1B, 2, 4, 9, 31 and 43. Only one of these, type 1B, corresponds to a type used in our study, and HeLa cells were much less susceptible to infection with that strain (Table 1). The results reported by Lewis and Kennett (13) are difficult to interpret, since they did not type the rhinovirus isolates. These authors suggested that the higher isolation rate in HeLa might be explained by the presence of certain rhinovirus serotypes more easily isolated in HeLa cells, a conclusion that would agree with the one we offer in this paper. However, the sensitivity of their diploid cell strain is unknown.

Variation of susceptibility of cell cultures to rhinoviruses has plagued efforts to isolate rhinoviruses. The isolation efficiency of any cell culture system cannot be measured against an objective measure such as serology because of the multiplicity of serotypes. Thus, it is difficult to compare these results with those of others (13, 17), not only because cell lines vary widely in susceptibility to rhinoviruses, but also because of the possibility that certain rhinoviruses may be different in their host cell tropisms. The latter possibility suggests that a survey of human heteroploid cells for susceptibility to rhinoviruses should be promising, particularly in light of the findings that most human heteroploid cells (known to vary widely in their viral susceptibility) appear to be in fact HeLa cells (15). It is interesting to note that characteristics of sensitivity or insensitivity to rhinoviruses of heteroploid cell lines appear to be stable in cell lines even with 10 years of passage. For example, HeLa 229 was originally characterized as insensitive to rhinovirus type 2 (5). This report extends this finding to indicate that HeLa 229 is insensitive to many rhinoviruses. On the other hand, the HeLa M cell line has also been carried in serial passage through most of the 12 years between the previous report(s) and this presentation and still maintains sensitivity.

ACKNOWLEDGMENTS

We wish to thank Francisca Morales for excellent technical assistance. This investigation was supported in part by

Public Health Service grant AI 09775 from the National Institute of Allergy and Infectious Diseases and by training grant GM 019935 from the National Institute of General Medical Sciences.

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