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Evaluation of Mixed Cell Types and 5-Iodo-2′-Deoxyuridine Treatment upon Plaque Assay Titers of Human Enteric Viruses

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Four continuous cell lines, BGM, L-132, HEL-299, and RD, were compared both when cultured separately and as mixtures for use in plaque assay titrations of human adenovirus 1 and six human enterovirus serotypes. The effect of incubating these cell cultures in media containing 5-iodo-2′-deoxyuridine (IDU) prior to inoculation with virus was also studied. The use of mixed-cell cultures revealed cell line-dependent synergistic effects as well as inhibitory effects. These effects were strongly virus dependent. In particular, enterovirus 69 did not form plaques on any of the four cell lines when cultured independently. However, it did form plaques on nearly all of the cell lines when cultured as mixtures. Contrary to this effect, when BGM cells were used in combination with the other cell lines, plaque counts for adenovirus 1 were greatly reduced. The effect of IDU pretreatment was also virus and cell line specific and enabled some viruses to form plaques on cell lines when they otherwise would not. Overall, IDU pretreatment resulted in an approximate twofold increase in plaque titers over those obtained without treatment.

Despite improvements in concentration methodology for the recovery of viruses from processed water and wastewater samples, the actual isolation of viruses from such processed samples frequently remains a difficult task. This is due to the highly variable extent to which different virus types replicate in the various available host systems (2, 3, 7, 11, 12, 14). Recovery of some virus types, such as the common enteroviruses, is relatively simple, as most replicate in one or another of established cell lines. Nonetheless, for reasons of economy in terms of human effort, supplies, and processed sample material, it is helpful if a correct matching of cell line choice for the viruses present in any given sample can be achieved on the first attempt (13).

A variety of additives have been evaluated for use in cell culture media in efforts to broaden the natural susceptibilities of cell lines to different virus types. These additives include, but are not limited to, chymotrypsin (10, 17), DEAE dextran (5), erythrocyte extract (16), 5-iodo-2′-deoxyuridine (IDU; 1, 6, 8, 9, 15, 18), and $MgCl_2$ (19). Halogenated pyrimidine IDU has frequently been found to work well for this purpose, particularly in the case of adenoviruses. In a study in our laboratory (1) IDU not only increased the cell and virus susceptibility spectra but also decreased the time required for the development of viral cytopathogenic effects.

The objective of the present study was to evaluate the use of mixed-cell cultures in combination with IDU pretreatment for plaque titration of one human adenovirus serotype and several human enterovirus serotypes.

A previous comparative study conducted in this laboratory (1) showed that the BGM, RD, L-132, and HEL-299 continuous cell lines were more susceptible than a number of others to a wide range of human enteric viruses in terms of cytopathogenic effects. Therefore, in the present study these four cell lines and combinations of them were chosen as the most likely candidates to provide maximal plaquing.

MATERIALS AND METHODS

Cell cultures. The continuous cell lines RD, L-132, and HEL-299 were obtained from the American Type Culture Collection, Rockville, Md. BGM cells were obtained from D. Dahling, U.S. Environmental Protection Agency, Cincinnati, Ohio. The optimal growth medium differed for each cell line (1) and, as a result of this difference, stock cultures of each cell line were normally passaged in their own optimal growth media. Individual-cell monolayers to actually be used for virus assays were then prepared from these stocks and grown in a compromise medium which consisted of Eagle minimum essential medium supplemented with 15% fetal calf serum, 0.1% lactalbumin hydrolysate, and 1 mM sodium pyruvate. Individual cultures for use in plaque assays were prepared in 25-cm^2 tissue culture flasks with the compromise medium supplemented, when needed, with 50 μg of IDU per ml. Mixed-cell monolayers were prepared by suspending together in the compromise medium equal volumes of freshly trypsinized cells of the appropriate types and then seeding 5 ml of these mixed suspensions directly into culture flasks. L-132 cells were adjusted to 2.0×10^5 cells per ml, and the other three lines were adjusted to 3.0×10^5 cells per ml. L-132 cells were added at a lower concentration because they appeared to multiply faster than did the other lines. Thus, in this way the ratios of cells in the produced monolayers visually appeared to be more evenly balanced. The total number of cells added to each flask varied from 1.0×10^6 to 1.5×10^6 based upon the volume of L-132 cells added but in all cases was sufficient to yield confluent monolayers within 72 h. When mixed-cell monolayers were prepared, these cultures could clearly be discerned to consist of more than one cell type and appeared to be evenly dispersed.

Virus preparations and plaque assays. The viruses used in the study were human adenovirus 1 (strain Adenoid 71), coxsackievirus A3 (strain Olson), coxsackievirus A7 (strain AB IV [USSR]), coxsackievirus B3 (strain Nancy), echovirus 7 (strain Wallace), enterovirus 69 (strain Toluca-1), and poliovirus 1 (strain LSc 2ab). These were propagated individually in tube cultures (16- by 125-mm tissue culture tubes) of each cell line used in the study. Stock virus suspensions for plaque assays were prepared by individually inoculating the viruses into two tube cultures of each cell type (eight tubes total for each virus type). Viral replication was allowed to progress in each of the inoculated tubes until

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either 4+ cytopathogenic effects were noted or 7 days had elapsed regardless of cytopathogenic effects. At either stage the individual tubes were then frozen and thawed three times and centrifuged for 10 min at 450 × *g* to pellet cell debris, and the supernatants were pooled for each virus type to yield stock virus suspensions for plaque assays. Serial 10-fold dilutions were made from the resulting virus stocks, and these were inoculated into IDU-pretreated and untreated monolayers of each cell type which had first been washed twice with Hanks balanced salt solution. The inoculated monolayers were then incubated for 2 h at 37°C to allow the viruses to bind to the cells and initiate infection. During this incubation period the flasks containing the inoculated monolayers were tilted and rotated at 30-min intervals to redistribute the inoculum and to keep the monolayers evenly moist. This action appeared to be a requisite to maintain viability of the HEL-299 cells. The monolayers were each then overlaid with 10 ml of a warm (43°C) agar overlay medium containing, per liter, 875 ml of Eagle minimum essential medium, 20 ml of chicken serum, 20 ml of pancreatin (1% [wt/vol]), 20 ml of neutral red (0.75% [wt/vol]; molecular weight, 5×10^5), 30 ml of $NaHCO_3$ (7.5% [wt/vol]), 10 ml of skim milk (Difco Laboratories, Detroit, Mich.), 25 ml of $MgCl_2$ (1 M), and 10 g of purified agar (Oxoid Ltd., London, England). After the agar overlay medium had been allowed to harden on the monolayers, the flasks were inverted and incubated at 37°C. Plaques were counted daily until they coalesced or until no new plaques appeared.

For stock suspensions of each virus type, that dilution which provided the highest number of plaques on any cell line without their overlapping was chosen for use during comparative assays. Having thus determined an optimal dilution for each virus stock, we adjusted the entire volume of each stock to that dilution and froze aliquots at −75°C for replicate assays. The highest number of plaques which could be counted accurately was determined by the characteristic plaque size for each virus type and was fairly consistent between the cell lines. For example, the coxsackievirus and poliovirus plaques were 10 mm or greater in diameter after 3 days of incubation, enabling a maximal accurate accounting of only 8 to 10 plaques per 25-cm^2 culture flask. The adenovirus plaques attained a diameter of only 1 to 2 mm by days 12 to 14, enabling an accurate accounting of as many as 30 to 35 plaques per 25-cm^2 culture flask.

RESULTS

The titer of the optimal assay dilution varied for each virus type. For this reason the plaque titers between the virus types were too divergent to enable an easy, direct comparison. To facilitate their comparison, we proportionately adjusted the obtained plaque titers upward such that the highest value for each virus type was 39.7 PFU/ml. This was the highest actual titer recorded and had been obtained with coxsackievirus A3 on IDU-pretreated HEL-299 cells. The adjusted titers are presented in Table 1, and they represent the averages of two trials with 10 complete sets of replicates per trial. The titers for coxsackievirus A3 were not adjusted, as that virus type was chosen to represent the base line.

IDU pretreatment generally resulted in increased plaque titers. Coxsackievirus A7 was a sole exception in this regard, as plaque formation by this virus type was normally inhibited by IDU pretreatment.

Without IDU pretreatment enterovirus 69 did not produce plaques on either any single-cell-type monolayers or the BGM-RD-cell-type monolayers. Enterovirus 69 did, however, produce plaques without IDU pretreatment on all other combined-cell-type monolayers, indicating the expression of a synergistic or helper effect on the part of the cells.

Adenovirus 1 did not produce plaques on BGM- or RD-cell-type monolayers regardless of exposure to IDU. The virus did produce plaques on L-132-cell-type monolayers with IDU pretreatment and on HEL-299-cell-type monolayers both with and without IDU pretreatment. It should be noted that the adenovirus 1 plaque titer obtained with IDU pretreatment on the HEL-299 cell type was approximately one-half that obtained without IDU pretreatment. However, the combined presence of either RD or L-132 cells in cell monolayers with HEL-299 cells resulted in the production of at least an equivalent plaque titer on the IDU-pretreated cells as on the untreated cells. No plaques were produced when adenovirus 1 was inoculated into any mixed-cell monolayer which contained BGM cells unless IDU pretreatment was used, and even then plaques were produced only when the monolayers contained L-132 cells. Thus, BGM cells sometimes seemed to be inhibitory to plaque production by adenovirus 1. Even though adenovirus 1 did not produce plaques on RD-cell-type monolayers either with or without IDU pretreatment, RD cells did not demonstrate any inhibitory activity when present in combination with other cell types. BGM cells did not demonstrate inhibitory activity for any of the other virus types examined in this study.

Plaques were not produced when echovirus 7 was inoculated into either L-132- or HEL-299-cell-type monolayers unless the cells had been pretreated with IDU. The same was true when these two cell types were combined together. These cell types did not, however, demonstrate any inhibitory activity towards echovirus 7 plaque production. Likewise, although none of the four cell types yielded plaques from enterovirus 69 unless pretreated with IDU, none demonstrated any inhibitory activity towards enterovirus 69 plaque production when present in combination with other cell types.

The ratios of viral plaque titer in IDU-pretreated cell cultures versus untreated cell cultures (enhancement ratios) were calculated for each individual cell type and also for the cell combinations (Table 2). It can be seen that with the exception of coxsackievirus A7, IDU pretreatment usually resulted in an average two- to threefold increase in the plaque titer. These ratios do not, of course, take into consideration the dramatic result observed with those virus-cell type combinations for which no plaques were yielded without IDU pretreatment. For coxsackievirus A7, IDU pretreatment resulted in an average titer reduction of 40%. A very dramatic enhancement ratio of 34.4 occurred in the case of enterovirus 69 when assayed on BGM–L-132-cell-type monolayers.

From an examination of the plaque titer ratios and excepting the 34.4 value obtained for enterovirus 69 on the BGM–L-132 cell combination, there did not appear to be any particular virus type, individual cell type, or cell combination which yielded consistently and exceptionally high ratios. Likewise, from an examination of the plaque titers listed in Table 1, although in many cases mixed-cell cultures appeared to be more sensitive for virus titrations than were single-cell cultures, no one individual cell type or cell combination was superior for the entire spectrum of viruses tested.

DISCUSSION

The reason for the observed synergistic effect when enterovirus 69 was assayed in mixed-cell cultures is un-

TABLE 1. Effect of mixed-cell monolayers and IDU pretreatment on viral plaque formation

Cell type[a]	Treatment[b]	Adjusted titer[c] of: Adenovirus 1	Coxsackievirus A3	Coxsackievirus A7	Coxsackievirus B3	Echovirus 7	Enterovirus 69	Poliovirus 1
Single cell								
B	Untreated	0	2.1 ± 1.1	6.0 ± 2.3	22.9 ± 12.1	24.9 ± 12.6	0	21.6 ± 4.3
	Treated	0	12.1 ± 2.7	3.8 ± 2.3	39.7 ± 12.0	39.7 ± 11.9	32.9 ± 16.5	39.7 ± 9.3
R	Untreated	0	5.7 ± 1.6	10.7 ± 3.3	11.3 ± 3.8	15.4 ± 4.9	0	18.5 ± 8.8
	Treated	0	17.1 ± 3.6	2.5 ± 1.9	22.6 ± 7.2	31.9 ± 9.9	9.2 ± 7.6	39.7 ± 7.4
L	Untreated	0	4.2 ± 3.8	24.8 ± 4.2	5.2 ± 3.1	0	0	5.3 ± 5.9
	Treated	39.7 ± 7.8	9.0 ± 3.5	31.2 ± 5.2	8.1 ± 3.4	4.1 ± 3.7	8.7 ± 3.6	11.9 ± 5.6
H	Untreated	12.8 ± 5.6	22.0 ± 3.6	39.7 ± 12.4	7.2 ± 3.9	0	0	1.8 ± 2.2
	Treated	6.3 ± 4.1	39.7 ± 4.3	22.4 ± 5.6	23.8 ± 5.0	2.0 ± 2.7	9.2 ± 7.6	11.5 ± 5.3
Two cell								
BR	Untreated	0	6.5 ± 1.7	6.8 ± 1.2	19.4 ± 6.7	12.8 ± 5.9	0	31.3 ± 12.4
	Treated	0	12.7 ± 1.8	3.5 ± 2.6	33.7 ± 4.3	26.9 ± 7.6	15.0 ± 7.0	35.7 ± 10.4
BL	Untreated	0	9.2 ± 1.7	25.6 ± 4.6	12.2 ± 5.0	1.7 ± 1.9	1.0 ± 1.9	26.5 ± 7.9
	Treated	17.8 ± 4.4	16.1 ± 1.8	23.0 ± 3.9	20.3 ± 7.7	10.7 ± 4.3	34.4 ± 13.8	32.2 ± 9.5
BH	Untreated	0	11.5 ± 1.7	25.0 ± 1.5	16.8 ± 8.1	4.6 ± 3.5	27.1 ± 10.7	20.3 ± 9.1
	Treated	0	17.3 ± 2.0	12.2 ± 1.9	25.5 ± 8.3	9.3 ± 3.4	33.9 ± 13.7	34.0 ± 7.9
RL	Untreated	4.8 ± 2.9	8.5 ± 1.4	20.4 ± 3.0	9.9 ± 7.1	3.8 ± 3.2	9.2 ± 6.7	26.0 ± 9.4
	Treated	14.7 ± 3.6	16.0 ± 1.7	14.9 ± 2.7	20.3 ± 5.8	15.4 ± 5.2	25.7 ± 6.5	37.1 ± 12.9
RH	Untreated	1.5 ± 2.5	12.7 ± 4.2	20.0 ± 2.2	7.5 ± 4.5	6.4 ± 3.6	26.1 ± 13.7	18.5 ± 10.6
	Treated	1.5 ± 1.5	22.2 ± 2.1	7.2 ± 1.8	16.5 ± 3.7	13.3 ± 5.7	29.5 ± 11.3	35.3 ± 9.0
LH	Untreated	32.0 ± 10.0	10.9 ± 1.6	39.7 ± 3.8	9.0 ± 3.8	0	2.9 ± 4.4	9.7 ± 8.6
	Treated	30.4 ± 4.7	15.8 ± 2.5	29.4 ± 2.6	13.3 ± 4.7	2.9 ± 2.6	7.3 ± 7.3	6.2 ± 2.9
Three cell								
BRL	Untreated	0	8.8 ± 1.7	14.5 ± 3.2	11.0 ± 7.7	6.1 ± 3.5	8.2 ± 7.8	24.3 ± 8.2
	Treated	9.1 ± 4.2	15.7 ± 2.2	7.0 ± 1.4	18.0 ± 6.8	14.8 ± 7.1	36.3 ± 12.9	34.8 ± 10.8
BRH	Untreated	0	11.2 ± 2.1	16.1 ± 4.1	12.2 ± 9.7	10.4 ± 4.5	17.9 ± 10.8	17.2 ± 7.5
	Treated	0	20.8 ± 3.6	8.1 ± 2.2	22.0 ± 8.7	18.8 ± 8.1	29.5 ± 7.3	27.8 ± 11.9
BLH	Untreated	0	12.7 ± 2.2	31.3 ± 2.5	12.8 ± 5.1	1.7 ± 1.9	17.9 ± 6.5	19.0 ± 8.4
	Treated	12.3 ± 4.4	20.6 ± 2.9	22.3 ± 2.6	17.7 ± 5.3	6.1 ± 4.2	39.7 ± 13.9	37.5 ± 13.6
RLH	Untreated	2.7 ± 2.0	12.4 ± 1.7	31.9 ± 4.1	8.4 ± 4.2	3.2 ± 3.8	8.7 ± 7.4	20.7 ± 9.7
	Treated	10.5 ± 3.8	18.3 ± 3.7	17.0 ± 2.7	16.8 ± 7.4	9.6 ± 4.1	28.1 ± 13.1	25.6 ± 10.4
Four cell								
BRLH	Untreated	0	12.2 ± 2.4	19.4 ± 3.4	13.6 ± 5.7	4.1 ± 4.1	11.1 ± 5.8	19.4 ± 10.7
	Treated	8.7 ± 3.9	18.3 ± 2.7	13.0 ± 1.9	18.3 ± 6.5	13.9 ± 13.9	35.3 ± 15.9	31.4 ± 9.1

[a] B, BGM; R, RD; L, L-132; H, HEL-299. The four cell types were used to yield single-cell-type or mixed-cell type monolayer cultures. See Materials and Methods for details.

[b] Untreated monolayers were not treated with IDU prior to inoculation with virus. Treated monolayers were incubated in media containing 50 μg of IDU per ml for 72 h prior to inoculation with virus.

[c] Results of the plaque assays are reported as adjusted PFU per milliliter of stock virus suspension ± standard deviation. The adjusted values were derived from the average plaque titers obtained from two independent trials, each consisting of 10 complete sets of replicates, for every virus-cell type combination, adjusted upward proportionately on the basis of virus type such that the highest titer for each virus type was 39.7 PFU/ml (the highest titer naturally attained with any of the virus-cell type combinations).

known. Cocultivation techniques (4) are often used for the recovery of many virus types; however, these normally involve two cell lines which differ with respect to permissiveness for the virus in question. In such techniques the cell type used as a helper is one that allows full replication of the virus; this was not the case in our studies involving enterovirus 69, as neither cell line would support replication independently.

Our finding that in many cases mixed-cell cultures yielded higher plaque titers than did single-cell cultures indicates that mixed-cell cultures may be preferable for the isolation of certain virus types. A particularly good example of this is enterovirus 69. The results of the present study have confirmed a previous finding by one of us that IDU pretreatment can be a powerful tool in enteric virus assays. Perhaps even more important than our finding about the combined benefits of mixed-cell cultures and IDU pretreatment for virus assays is our discovery that the presence of some cell types in mixed-cell cultures may preclude the replication of certain viruses.

No factor which would account for the observed plaque titer reduction of coxsackievirus A7 caused by IDU pretreat-

TABLE 2. Plaque titer ratios of IDU-pretreated versus untreated cell monolayers

Cell type[a]	Ratio[b] for: Adenovirus 1	Coxsackievirus A3	Coxsackievirus A7	Coxsackievirus B3	Echovirus 7	Enterovirus 69	Poliovirus 1
Single cell							
B		5.8	0.6	1.7	1.6		1.8
R		3.0	0.2	2.0	2.1		2.1
L		2.1	1.3	1.6			2.2
H	0.5	1.8	0.6	3.3			6.5
Mean ± SD	0.5	3.2 ± 1.6	0.7 ± 0.4	2.1 ± 0.7	1.8 ± 0.2		3.1 ± 1.9
Two cell							
BR		2.0	0.5	1.7	2.1		1.1
BL		1.8	0.9	1.7	6.2	34.4	1.2
BH		1.5	0.5	1.5	2.0	1.2	1.7
RL	3.1	1.9	0.7	2.1	4.1	2.8	1.4
RH	1.0	1.7	0.4	2.2	2.1	1.1	1.9
LH	0.9	1.4	0.7	1.5		2.5	0.6
Mean ± SD	1.7 ± 1.0	1.7 ± 0.2	0.6 ± 0.2	1.8 ± 0.3	3.3 ± 1.7	8.4 ± 13.0[c], 1.9 ± 0.8[d]	1.3 ± 0.4
Three cell							
BRL		1.8	0.5	1.6	2.4	4.4	1.4
BRH		1.9	0.5	1.8	1.8	1.7	1.6
BLH		1.6	0.7	1.4	3.5	2.2	2.0
RLH	3.9	1.5	0.5	2.8	3.0	3.2	1.2
Mean ± SD	3.9	1.7 ± 0.2	0.6 ± 0.1	1.9 ± 0.5	2.7 ± 0.6	2.9 ± 1.0	1.5 ± 0.3
Four cell							
BRLH		1.5	0.7	1.3	3.4	3.2	1.6
Overall mean ± SD	1.9 ± 1.4	2.2 ± 1.1	0.6 ± 0.2	1.8 ± 0.5	2.8 ± 1.3	5.7 ± 9.6[c], 2.5 ± 1.0[d]	1.9 ± 1.3

[a] B, BGM; R, RD; L, L-132; H, HEL-299. See Materials and Methods for details.
[b] IDU-pretreated monolayers were incubated in media containing 50 μg of IDU per ml for 72 h prior to inoculation with virus. Untreated monolayers were not treated with IDU prior to inoculation with virus. The ratio is that of plaque titers obtained for IDU-pretreated versus control cell monolayers, as listed in Table 1, rounded to the nearest 0.1.
[c] Including the 34.4 ratio for the BL combination.
[d] Excluding the 34.4 ratio for the BL combination.

ment was apparent. However, based upon our previous study dealing with the time of appearance of CPE (1), this result was not unexpected. During the previous study, the majority of group A coxsackieviruses produced CPE in IDU-pretreated cells either at the same rate as or more slowly than in untreated cells.

During the present study, there were only five instances in which mixed-cell cultures yielded higher plaque titers than those obtained with the single-cell cultures. Even in these instances the counts were not appreciably higher. Therefore, it would appear that the mixing of virally permissive cells with nonpermissive cells is not a good answer to the problem of using a single cell type for virus recovery and enumeration. Indeed, for adenovirus 1 recovery, the inclusion of BGM cells in the mixtures caused a titer reduction of greater than 50%.

Although the plaque titer results obtained from the mixed-cell cultures were disappointing, the observed effects attributable to IDU incorporation were not. Plaque production was increased in IDU-pretreated cells by an average of 63% even when allowing for the negative effect observed for coxsackievirus A7. If results obtained with this virus were excluded from the calculations, the average plaque titer increase in IDU-pretreated versus untreated cells would be 101%. No attempt was made to increase plaquing efficiencies by altering the ingredients of the overlay medium.

The utopian goal of using a single-cell culture or cell treatment to enable replication of all enteric viruses still seems elusive. However, based upon the experiments in the present study and our previous study (1), it would seem that IDU-pretreatment of cells can be of great value in increasing the enteric virus recovery potential of existing cell culture systems.

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