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Comparative Studies of the Regeneration of HeLa Cell Receptors for Poliovirus T1 and Coxsackievirus B3[1]

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Enterovirus receptors of live HeLa cells have been shown to reappear after inactivation by proteolytic enzymes, provided the cells are incubated at 37 C in a nutritionally adequate medium. Regeneration of receptor activity for poliovirus T1 occurred at a significantly faster rate than for coxsackievirus B3. The regenerative process for both types of receptors studied evidently required an active process of protein synthesis, since it was found that reappearance of receptor activity was inhibited by streptovitacin A, puromycin, and actinomycin D. Substitution of *p*-fluorophenylalanine for the naturally occurring amino acid, at concentrations which inhibited virus synthesis, was without effect on regeneration of receptor activity. It is anticipated that these findings will aid in the study of the biosynthesis and subsequent chemical characterization of viral receptors of living host cells.

Zajac and Crowell (30, 31) have reported that the presence of receptor activity for both poliovirus T1 and coxsackievirus B3 is limited to the surface of viable HeLa cells and that these receptors can be inactivated selectively by trypsin and chymotrypsin. In addition, enzyme-treated cells were shown to be capable of regeneration of enterovirus receptor activity in the absence of cell division, provided the cells were returned to growth medium and incubated at 37 C. In many aspects, these latter observations were analogous to those found to occur for myxovirus receptors of host cells (1, 8, 18, 19, 26, 27) and for encephalomyocarditis virus receptors (14) after treatment with receptor destroying enzyme. The intracellular events controlling the regeneration of viral receptors which have been inactivated by enzymes, however, have not been reported in detail. This communication presents evidence that the regeneration of receptor activity by HeLa cells for two enteroviruses occurs at significantly different rates and that both regenerative processes require an active synthesis of cellular proteins.

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MATERIALS AND METHODS

HeLa cells. Cells designated HeLa (JJH) were cultured as monolayers in growth medium containing 10% calf serum (GM-CaS10), as described elsewhere (30).

Virus strains. Poliovirus T1 and coxsackievirus B3 strains used in this investigation and the method of plaque assay were described previously (5).

Receptor activity of HeLa cells. HeLa cell receptor activity was expressed in terms of the amount of virus which became attached to cells after incubation of 10^6 plaque-forming units (PFU) of virus per ml with a suspension of 10^7 cells per ml for 1 hr at 37 C. The amount of virus which was attached to cells was determined by the difference between the amounts of free virus found and the input virus. A difference of 10% was not considered significant. As a measure of initial virus content, a virus control tube containing the virus inoculum and growth medium, in place of the cell suspension, was included in each experiment.

Inactivation of HeLa cell receptor activity. Viral receptors of HeLa cells were inactivated by use of freshly prepared solutions of crystalline preparations of either or both trypsin and chymotrypsin (30). Cells suspended in Hank's balanced salt solution (BSS) were added to an equal volume of enzyme solution to give a final concentration of 3×10^6 cells/ml and an enzyme concentration of 1%, except when indicated otherwise. The enzyme-cell suspension was incubated at 37 C for 1 hr in a shaker-water bath. The *p*H of the suspension was maintained at approximately 7.8 by the addition of a few drops of 3%

$NaHCO_3$ at intervals as needed. After enzyme treatment, the cells were enumerated, diluted approximately twofold with BSS, and collected by centrifugation at 900 × *g* for 20 min in the cold. In this way, known numbers of enzyme-treated cells were obtained for use in studying regeneration of viral receptors under different conditions.

Determination of receptor regeneration. Enzyme-treated HeLa cells were resuspended, except when stated otherwise, in growth medium containing twice-concentrated Eagle's complete amino acid-vitamin mixture, supplemented with 20% calf serum [GM (2×)]. The cells were incubated at 37 C at a final concentration of 10^6 cells/ml and were shaken periodically. At intervals, samples of 10^7 cells were removed, washed, and tested for their ability to attach poliovirus T1 and coxsackievirus B3. To measure receptor regeneration for periods which exceeded 8 hr, additional samples of 2.5×10^7 treated cells were distributed to new 0.95-liter prescription bottles for incubation in a stationary, horizontal position at 37 C. At intervals up to 48 hr, the fluid phase of a replicate culture was decanted, the cell monolayer was gently washed twice with BSS, and the attached cells were removed from the glass surface with the aid of 0.02% ethylenediaminetetraacetic acid and a rubber scraper. The cells were washed and enumerated, and samples of cells were tested for ability to attach virus. Normal cells, which were processed concurrently, although not treated with enzyme, served as a control.

Metabolic inhibitors and enzymes. Solutions of metabolic inhibitors and enzymes were freshly prepared for each experiment. Inhibitors were dissolved in BSS to the desired concentration and were kept in the dark at 4 C prior to being used. Streptovitacin A was kindly provided by The Upjohn Co., Kalamazoo, Mich. Puromycin dihydrochloride and *p*-fluorophenylalanine (FPA) were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, and Sigma Chemical Co., St. Louis, Mo., respectively. Actinomycin D was graciously donated by Merck & Co., Inc., Rahway, N.J. The proteolytic enzymes, trypsin and α-chymotrypsin were purchased as crystalline preparations from Worthington Biochemical Corp., Freehold, N.J. (30).

Evaluation of metabolic inhibitor activity as measured by inhibition of virus synthesis. Inhibition of synthesis of infectious virus was used as a convenient criterion for assessing the effectiveness of the various concentrations of metabolic inhibitors used in this study. Tubes containing pellets of 10^7 enzyme-treated or untreated cells were suspended in 2 ml of either poliovirus T1 or coxsackievirus B3 (10^8 PFU/ml), depending on which enzyme was used; e.g., coxsackievirus B3 was used for trypsinized cells since trypsin does not inactivate the B3 receptors, and poliovirus was used for chymotrypsinized cells for a similar reason. Virus was allowed to attach for 1 hr at 37 C, at which time residual free virus was reduced by washing the cells three times with 10-ml amounts of BSS, keeping a sample of the third wash as a measure of background virus. Replicate samples of infected cells were resuspended in GM (2×) containing different concentrations of the inhibitor to be tested. Treated and untreated cells receiving no inhibitor served as controls. The virus-infected cell suspensions were incubated 6 hr at 37 C for the testing of streptovitacin A and puromycin, whereas a 24-hr period was employed for *p*-fluorophenylalanine. To release intracellular virus, the cells were subjected to seven cycles of freezing and thawing by alternately placing the samples in liquid nitrogen and in a water bath at 37 C, and vigorously mixing the thawed samples on a Vortex Junior Mixer (Scientific Industries, Inc., Queens Village, N.Y.). The supernatant fluid containing the total virus yield was harvested after centrifugation at 900 × *g* for 20 min and was assayed for amount of infectious virus. The minimal concentration of inhibitor which resulted in almost complete repression of viral synthesis was employed in the study for assessing effects on receptor regeneration.

RESULTS

Comparative rates of receptor regeneration. In initial studies, the rates of regeneration of poliovirus T1 and coxsackievirus B3 receptor activities of HeLa cells were measured for cells treated previously with a combination of 1% trypsin and 1% chymotrypsin to inactivate the respective viral receptors. Enzyme-treated cells were washed in BSS, enumerated, resuspended in GM (2×) at a final cell concentration of 10^6/ml, and incubated at 37 C. At intervals up to 48 hr, samples were removed, and the cells were tested for reappearance of viral receptor activity.

The results of a representative experiment (Fig. 1) indicate that HeLa cells regained their reactivity for poliovirus T1 much more rapidly than that for coxsackievirus B3. Similar results have been obtained in repeated experiments and also when cells were treated with either trypsin or chymotrypsin independently.

Requirement of protein synthesis for receptor regeneration. The results of numerous studies have suggested that enterovirus receptors of HeLa cells are composed principally of lipoprotein (11, 20), with the protein moiety most likely providing the functional sites for virus attachment (11). The intracellular events leading to the biosynthesis of receptor material, however, have not been studied. The next experiments to be described were designed to evaluate the dependency of enterovirus receptor regeneration on cellular protein synthesis.

Streptovitacin A, which has been shown to be an effective and rapidly acting inhibitor of protein synthesis of cells in culture (6, 25), was incorporated into the growth medium used for study of HeLa cells regenerating receptors for poliovirus T1. Streptovitacin A was used at a final concentration of 5 μg per ml of medium. This concentration of inhibitor was shown in preliminary experiments to inhibit completely the synthesis of

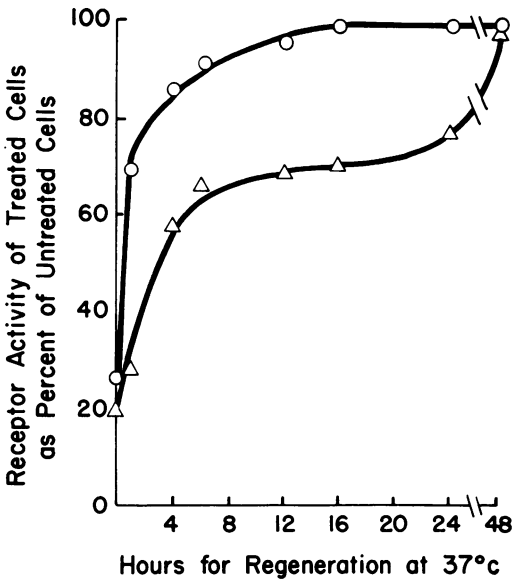

FIG. 1. *Comparative rates of regeneration of receptor activity for poliovirus T1 (○) and coxsackievirus B3 (△) by enzyme-treated HeLa cells.*

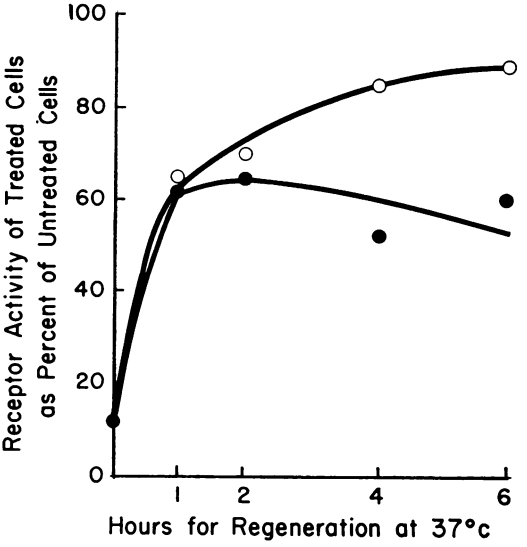

FIG. 2. *Effect of streptovitacin A (5 μg/ml) on the regeneration of poliovirus T1 receptor activity of HeLa cells (●). Regeneration in medium without inhibitor (○).*

TABLE 1. *Inhibition of coxsackievirus B3 replication by streptovitacin A (5 μg/ml)*

Cell sample	Coxsackievirus B3 titer (PFU/ml)[a]	
	Initial	6 hr
Untreated	1.0×10^3	1.2×10^6
Trypsin-treated	1.7×10^3	1.0×10^6
Trypsin-treated streptovitacin A	1.9×10^3	2.0×10^3

[a] Total infectious virus present was determined after disruption of cells.

coxsackievirus by trypsinized HeLa cells (Table 1). In a typical receptor regeneration experiment, samples of trypsin-treated cells, which had been placed in GM (2×) at 37 C in the presence or absence of streptovitacin A were removed at intervals and were tested for recovery of ability to attach poliovirus T1.

The results of a representative experiment presented in Fig. 2 indicate that the reappearance of receptor activity by cells incubated in the presence of streptovitacin A approximated that of the control during the initial hour; thereafter, however, the regeneration process was inhibited significantly. Since streptovitacin A employed even in higher concentrations did not inhibit the amount of receptor regeneration occurring during the 1st hr of incubation, it was necessary to determine whether the early appearance of receptor activity was due to a rapid synthesis of receptors prior to effective inhibition of protein synthesis or whether the regenerative process immediately after trypsin treatment occurred independently of protein synthesis. To resolve between these alternative possibilities, HeLa cells inactivated previously with trypsin were permitted to regenerate in the presence of streptovitacin A for 1 hr, as before, at which time they were retrypsinized to inactivate the newly recovered poliovirus receptor activity. After the second enzyme treatment, the cells were washed, transferred to fresh GM (2×) containing streptovitacin A, and were incubated. Samples of cells were removed periodically and tested for receptor activity. As a control, cells doubly treated with enzyme and incubated in the absence of streptovitacin A were tested in parallel.

The control cells (Fig. 3), after a second enzyme treatment, regenerated receptor activity at a rate similar to that shown previously for cells treated only once (cf. Fig. 2). However, a more pronounced inhibition of receptor regeneration by streptovitacin A was noted after the second trypsinization. The inhibition of receptor regeneration was shown to be reversible, since cells washed free of inhibitor and placed in inhibitor-free medium at 6 hr regained almost complete reactivity for poliovirus T1 within the next 12 hr. Additional experiments showed that the double treatment of cells with enzyme was not required

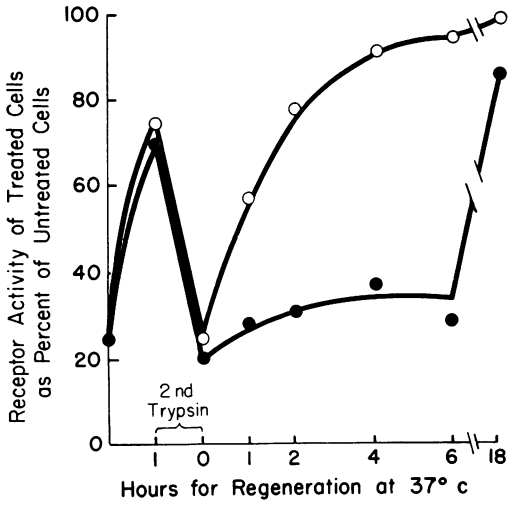

FIG. 3. *Effect of streptovitacin A (5 μg/ml) on the regeneration of poliovirus T1 receptor activity of HeLa cells which were doubly trypsinized (●). Regeneration in medium without inhibitor (○).*

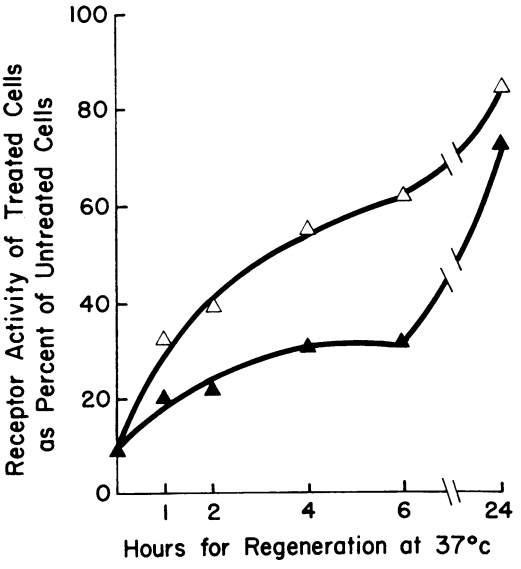

FIG. 4. *Effect of streptovitacin A (5 μg/ml) on the regeneration of coxsackievirus B3 receptor activity of HeLa cells (▲). Regeneration in medium without inhibitor (△).*

for inhibition of regeneration, provided cells were pretreated with streptovitacin A for 1 hr prior to trypsinization in the presence of the inhibitor. Evidently, the fast rate of receptor regeneration was not due to the rapid accumulation of endogenous preformed receptor precursors, but to a rapid de novo synthesis of poliovirus receptors. It is reasonable to assume that some time must be allowed to obtain effective inhibition of protein synthesis by streptovitacin A.

Chymotrypsin-treated HeLa cells were treated also for their capacity to regenerate receptors for coxsackievirus B3 in the presence of streptovitacin A at a concentration of 5 μg/ml. The results of this experiment (Fig. 4) revealed that coxsackievirus B3 receptors failed to regenerate when cellular protein synthesis was inhibited. Inhibition of this regenerative process was also shown to be reversible after 6 hr when the cells were returned to fresh medium devoid of streptovitacin A.

Puromycin, a more widely used inhibitor of protein synthesis, was also tested in experiments of design similar to those described previously for streptovitacin A. At a level of 5 μg/ml, puromycin also showed a reversible type of inhibition of poliovirus T1 receptor regeneration, to verify the results obtained with streptovitacin A.

In an attempt to determine whether synthesis of messenger ribonucleic acid (mRNA) also was needed for receptor regeneration, experiments were carried out in which actinomycin D was tested for inhibitory activity (22). Preliminary experiments revealed that regeneration of poliovirus T1 receptor activity was inhibited significantly by as little as 1.0 μg of actinomycin D per ml when preincubated with HeLa cells for 2 to 6 hr prior to trypsinization and when actinomycin D was included in the regeneration medium. In control experiments, actinomycin D included in GM (2×) at a final concentration of 1.0 μg/ml showed minimal cytotoxic activity at 8 hr, with generalized rounding of cells after 14 hr of incubation as visualized by microscopic observation of cells in monolayers. This same concentration of actinomycin D also was shown to inhibit vaccinia virus synthesis by 99%.

TABLE 2. *Effect of actinomycin D (1.0 μg/ml) on the regeneration of HeLa cell receptor activity for coxsackievirus B3 and poliovirus T1*

Receptor	Incubation (hr)	Amt of regeneration (per cent of untreated cells)	
		Normal	Actinomycin D
Coxsackievirus B3	0	5	5
	3	31	18
	6	47	21
Poliovirus T1[a]	0	0	3
	2	61	22
	4	71	33

[a] Double trypsinization procedure.

TABLE 3. *Effect of temperature on the regeneration of HeLa cell receptor activity for coxsackievirus B3 and poliovirus T1*

Receptor	Incubation (hr)	Amt of regeneration (per cent of untreated cells)		
		4 C	24 C	37 C
Coxsackievirus B3	0	5	5	5
	3	8	7	40
	6	6	22	47
Poliovirus T1	0	0	2	0
	2	2	24	61
	4	20	30	71

In a final experiment, replicate monolayer cultures of HeLa cells were treated with actinomycin D at a final concentration of 1.0 μg/ml for 2 hr at 37 C prior to inactivation of receptors by either trypsin or chymotrypsin. The inhibitor was present also during the period of enzyme treatment. After two washes with BSS, the cells were transferred to GM (2×) containing actinomycin D and were assayed for receptor activity at intervals of incubation at 37 C.

The results presented in Table 2 reveal that a significant reduction in regeneration of receptor activity for both viruses was produced by actinomycin D. Whether these results indicate that receptor regeneration is dependent on the synthesis of new mRNA cannot be stated with certainty, since, as reported recently (3, 12, 16), actinomycin D may inhibit additional cellular activities.

Effect of temperature on receptor regeneration. It was shown previously (30) that regeneration of coxsackievirus B3 receptor activity was repressed when chymotrypsin-treated HeLa cells were incubated at 4 C. Detailed studies of the regeneration of poliovirus receptors were not given. In the present study, cells were examined for their ability to regenerate receptors for both poliovirus T1 and coxsackievirus B3 at 4, 24, and 37 C after dual inactivation of receptors by trypsin and chymotrypsin. If protein synthesis were required for receptor biosynthesis, then low temperature would be expected to inhibit this cellular activity.

Replicate samples of HeLa cells, pretreated with enzymes, were incubated for 1 hr at 4, 24, and 37 C to allow residual receptor activity to be expressed. The cells were retreated and incubated in GM (2×) at the respective temperatures for receptor regeneration. The data obtained from this experiment (Table 3) show that, of the temperatures tested, 37 C was optimal for regeneration of both viral receptors.

Effect of an amino acid analogue on receptor regeneration. Since protein synthesis was found to be essential for regeneration of viral receptors, the final set of experiments were performed to determine whether the amino acid analogue, FPA, would inhibit these systems. Although FPA is known to depress cellular protein synthesis of HeLa cells when used in very high concentrations (> 300 μg/ml) (10), at lower concentrations, in phenylalanine-deficient medium, the analogue is very often incorporated into protein in place of the natural amino acid, resulting in nonfunctional proteins (23). It was for this latter reason that FPA was selected for testing. It was necessary, however, first to determine whether the cells derived the materials needed for the synthesis of receptor substance from accumulated intracellular nutrients or from the surrounding growth medium. Cells inactivated by double trypsinization were incubated in BSS and tested periodically for recovery of receptor activity for poliovirus T1. At 6 hr, the cells were washed and resuspended in GM (2×) for an additional 18 hr. Cells incubated in GM (2×) throughout the entire experiment served as control.

The data presented in Fig. 5 illustrate that HeLa cells require extracellular nutrients for the biosynthesis of poliovirus receptors. Cells suspended in BSS did not regenerate receptor activity, although the cells were fully competent as shown by their recovery of receptors when returned to complete medium after 6 hr.

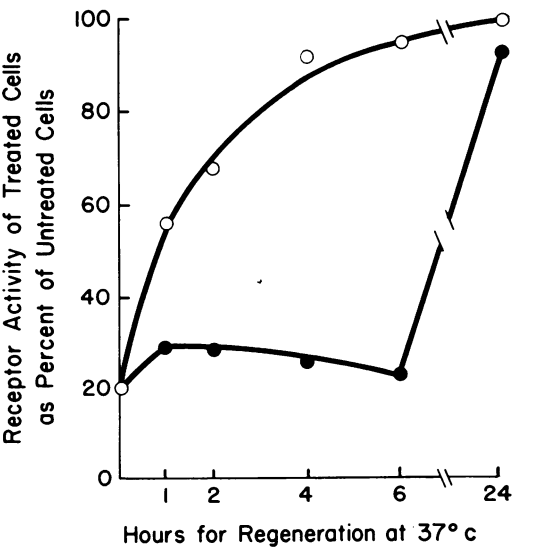

FIG. 5. *Inability of HeLa cells to regenerate receptor activity for poliovirus T1 in a nutritionally deficient medium* (●). *Regeneration in complete medium* (○).

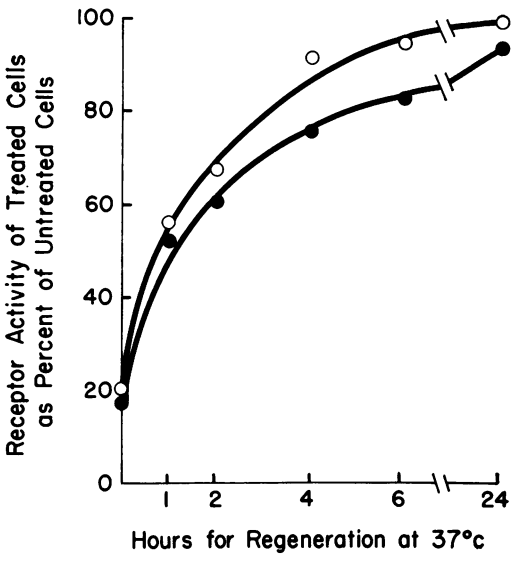

FIG. 6. *Effect of p-fluorophenylalanine (100 μg/ml) on the regeneration of poliovirus T1 receptor activity of HeLa cells (●). Regeneration in medium without inhibitor (○).*

In the following experiment, HeLa cells previously starved for 12 hr to exhaust the intracellular pool of phenylalanine were twice trypsin-treated, washed, and resuspended in phenylalanine-deficient medium containing FPA at a final concentration of 100 μg/ml. The calf serum employed in the growth medium had been dialyzed exhaustively against saline and found to contain no detectable peak of phenylalanine as determined by the Technicon autoanalyzer. Cells incubated in GM lacking FPA were tested in parallel. The results represented in Fig. 6 indicate that the regeneration of receptor activity for poliovirus T1 was not appreciably inhibited by FPA when compared to control cells. A slight inhibition of protein synthesis at this relatively high concentration of FPA might account for the failure of both curves to be superposable. Experiments of similar design in which FPA was tested at levels as low as 5 μg/ml showed no inhibition of regeneration.

Table 4 shows a similar lack of effect of FPA on the regeneration of coxsackievirus B3 receptors. After 24 hr of incubation in GM (2×), chymotrypsin-treated cells regenerated 85% of the activity possessed by untreated cells. An equivalent recovery was found for cells incubated in phenylalanine-deficient GM (2×) regardless of whether FPA was present or absent.

It has been shown (13, 17) that the synthesis of infectious poliovirus was inhibited by FPA at concentrations as low as 4 to 5 μg/ml. Virus, which

TABLE 4. *Effect of FPA (100 μg/ml) on the regeneration of receptor activity for coxsackievirus B3 by HeLa cells*

Cell sample	Amt of regeneration (per cent of untreated cells)
Complete GM (2×)[a]	85
Phenylalanine-deficient GM (2×)	86
Phenylalanine-deficient GM (2×) + FPA	86

[a] See Materials and Methods for composition of growth medium GM (2×).

TABLE 5. *Inhibition of poliovirus T1 replication by FPA (5 μg/ml)*

Cell sample	Poliovirus T1 titer (PFU/ml)[a]	
	Initial	24 hr
Untreated	1.0×10^3	3.1×10^6
Chymotrypsin-treated	1.3×10^3	1.3×10^6
Chymotrypsin-treated + FPA	2.1×10^3	4.0×10^3

[a] Total infectious virus present was determined after disruption of cells.

was formed under these conditions, was found to be structurally altered, possibly a reflection of the replacement of FPA for phenylalanine in capsid specific peptides (13). As a control for the present experiments, the synthesis of poliovirus T1 by chymotrypsin-treated cells in the presence and absence of 5 μg/ml of FPA was investigated. The results (Table 5) showed no significant synthesis of infectious virus occurred when FPA was present at a relatively low concentration in the medium. Thus, it was concluded that if phenylalanine were present in the receptor structures, it was not critically involved in the active sites for attachment of either poliovirus T1 or coxsackievirus B3.

DISCUSSION

Although HeLa cells were made equally refractory to poliovirus T1 and coxsackievirus B3 attachment by the combined action of trypsin and chymotrypsin, a significant difference was noted in the rates of regeneration of receptor activity for these two viruses. Receptor activity for poliovirus T1 regenerated to half of maximum within the 1st hr postenzyme treatment, whereas an equivalent recovery of receptor activity for coxsackievirus B3 required approximately 3 to 4 hr. The slower kinetics of regeneration of coxsackie-

virus B3 receptors by HeLa cells may reflect (i) a more complex receptor structure, (ii) the presence of more receptors per cell (R. L. Crowell, Bacteriol. Proc., p. 140, 1967), or (iii) a more extensive damage to the cell surface beyond that of specific receptor inactivation. A valid interpretation of the differences in rates of regeneration of the two viral receptors must await the acquisition of additional information.

The mechanism of the inactivation of enterovirus receptor activity by proteolytic enzymes is unknown. The fact that proteolytic enzymes remove significant amounts of material from mammalian cells is well documented (2, 9, 21, 24, 28, 29). In agreement with Philipson et al. (20), however, we have been unable to recover biologically active viral receptors released from cells at intervals of incubation with either trypsin or chymotrypsin, even when residual enzymes were removed by passage through Sephadex G-75 prior to the testing of fractions for receptor activity.

If the receptors under study were degraded and released from the cell surface by action of proteolytic enzymes, it was conceivable that a cellular repair mechanism (regeneration) would require either a de novo biosynthesis of these surface components or perhaps an unfolding of the cell surface to expose hidden receptors. This latter possibility has been considered to be unlikely since enzyme-treated cells placed in hypotonic solutions to expand the surface membrane failed to attach increased amounts of virus (*unpublished data*). The findings that inhibitors of protein synthesis, streptovitacin A, puromycin, and actinomycin D prevented regeneration of receptors and that cells failed to attain significant recovery of receptor activity when incubated at low temperatures or in nutritionally deficient medium, all supported the hypothesis that new biosynthesis of surface components was essential to recovery of the receptor activity.

Although protein synthesis was found to be necessary for receptor regeneration, FPA did not inhibit significantly this cellular activity. It should be recalled that FPA in much lower concentrations was nevertheless very effective in inhibiting synthesis of infectious virus. Evidently, if phenylalanine were present in the receptors studied, it is unlikely to be located at the active site for attaching virus.

That the chemical composition of enterovirus receptors of HeLa cells are lipoprotein structures has been suggested by Holland and Hoyer (11). Philipson and co-workers (20) found that by extracting erythrocyte membranes with a butanol-water mixture they could recover a complex substance in the aqueous phase which possessed receptor activity for hemagglutinating viruses. This receptor substance was found to be composed of protein, lipid, and carbohydrate. Although the results of the studies reported here indicate that protein synthesis is required for receptor regeneration by HeLa cells, this information does not necessarily mean that these receptors are protein, since protein synthesis may also influence the formation of carbohydrate containing cellular substance (7, 15).

The results reported herein, together with the recent finding by L. C. McLaren (*personal communication*) that enterovirus receptor activities of HeLa cells can be extracted in soluble form with Triton X-100, are proving useful in studying the biosynthesis and the chemical nature of the viral receptors of living host cells.

ACKNOWLEDGMENTS

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