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Infectivity of Poliovirus and Its Nucleic Acid for Dehydrated HeLa Cell Monolayers

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ABSTRACT

SMULL, CHRISTINE E. (The Geisinger Medical Center, Danville, Pa.), AND E. H. LUDWIG. Infectivity of poliovirus and its nucleic acid for dehydrated HeLa cell monolayers. J. Bacteriol. 89:52-57. 1965.—A study was made of the infectivity of poliovirus ribonucleic acid (RNA), applied in various diluents, and of poliovirus, on cell monolayers which were washed free from their nutrient medium and allowed to dehydrate for periods up to 3 days prior to inoculation. The plaque formation of poliovirus RNA, applied in isotonic diluent, was greatly increased when assayed on cell monolayers dehydrated for certain periods of time. Conditions are described for bringing about optimal plaque formation by this means, and evidence is presented which strongly indicates that dehydration of the cell is an important factor in the increase in plaque formation of the poliovirus RNA. The plaque formation of poliovirus RNA in certain hypertonic diluents and in a basic protein-containing diluent was also improved with the use of dehydrated cell monolayers, whereas the plaque formation of poliovirus varied very little when assayed on cell monolayers dehydrated for periods up to 2 days. Under certain conditions, the size of the plaques increased as the dehydration time of the cell monolayers was increased. This was true with plaques initiated either by poliovirus or the nucleic acid. Further investigation in these cases revealed that virus production at given times was considerably greater in the dehydrated than in the undried cell monolayers.

The infectivity of poliovirus ribonucleic acid (RNA) is normally so low that methods have been devised for enhancing its infectivity in tissue culture. Methods for increasing plaque formation include the use of hypertonic solutions (Alexander et al., 1958; Boeyé, 1959; Holland et al., 1960; Ellem and Colter, 1961), calcium-depleted cells and certain poorly water-soluble substances (Dubes and Klingler, 1961), and basic proteins (Smull, Mallette, and Ludwig, 1961).

Procedures for infection of cell monolayers with poliovirus RNA generally make use of cells washed free from their nutrient medium and inoculated shortly thereafter. The present paper reports a study of the infectivity of poliovirus and its nucleic acid with the use of cell monolayers which, after washing, were allowed to drain for periods up to 3 days prior to inoculation. In this paper, such cell monolayers have been designated as "dehydrated."

MATERIALS AND METHODS

Cell cultures, virus source, and preparation of infectious RNA. Pools of type 1 poliovirus were prepared in monkey heart cell cultures, and assays of infectivity of poliovirus and poliovirus RNA were made in HeLa cell monolayers. The RNA was prepared by a modification of the phenol method of Gierer and Schramm (1956). The method of cell culture, preparation of virus pools, and extraction and storage of the RNA were described previously (Smull and Ludwig, 1962).

Preparation of HeLa cell monolayers and assay of infectivity of RNA. HeLa cell monolayers in 3-oz prescription bottles were washed twice with 0.14 M NaCl and then allowed to drain at room temperature with the bottles capped and in an upright position. After a variable period of time, usually 1 day, the residual wash fluid which had accumulated at the bottom of the bottle was removed with a capillary pipette, and the dehydrated cell monolayers were then inoculated with 0.1 ml of an RNA preparation previously diluted in 0.14 M NaCl. After 2 to 5 min at room temperature for adsorption, 10 ml of overlay medium were added, and the cultures were incubated at 36 C for 2 to 3 days. The overlay medium was then removed, and the cell monolayers were stained with crystal violet (Holland and McLaren, 1959) for enumeration of plaques.

When ethyl alcohol was used, the cell monolayers were prepared as described above and, in addition, were treated with 0.2 ml of an appropriate ethyl alcohol solution after the preliminary cell washing. Any excess solution was removed with a capillary pipette immediately prior to inoculation of the RNA.

Comparison of virus production in dehydrated and undried cell monolayers. One half of a set of 1-day-old cell monolayers was allowed to dehydrate as usual for 24 hr; the other half of the set continued to incubate at 36 C with the nutrient medium. At the end of the dehydration period, all the cell monolayers were inoculated with 100 $TCID_{50}$ of poliovirus in a 0.1-ml suspension; 8 ml of cell maintenance medium were then added to each bottle. The cell maintenance medium was Medium 199 (Difco) diluted 1:3 in Hanks' balanced salt solution. After incubation at 36 C for various periods, the virus cultures were frozen, thawed, and then assayed for infectivity by plaque count on cell monolayers.

Histone. Histone (calf thymus nuclei), obtained from General Biochemicals Corp., Chagrin Falls, Ohio, was prepared and used as described previously (Ludwig and Smull, 1963).

RESULTS

Experiments with poliovirus RNA applied in isotonic diluent. The infectivity of the poliovirus RNA preparations used in this study was such that only occasional plaque formation was obtained when assay was made, in isotonic diluent, on freshly washed cell monolayers. But it was found that the same RNA preparations gave a somewhat increased and more regular plaque response when assayed on cell monolayers which were allowed to drain (dehydrate) at room temperature for several hours prior to inoculation. A further investigation was then made of this finding by use of cell monolayers dehydrated for periods up to 3 days. The study included experiments in which the caps were kept on the bottles and in which they were removed from the bottles during the dehydration of the cell monolayers.

With the use of cell monolayers dehydrated with the caps on the bottles, it was found that the plaque formation slowly increased with an increased dehydration time, reaching a maximum, usually, with cell monolayers dehydrated approximately 1 to 2 days (Table 1). Under optimal conditions, the infectivity of the RNA was 10^4 or more plaque-forming units (PFU) per ml. This represented an increase of approximately 100-fold over that obtained with the same preparations on undried cell monolayers. The efficiency of infection of the RNA under optimal conditions was approximately 0.001% of that of the whole virus preparation. Following the period of maximal plaque formation, the plaque number decreased slowly until again only occasional plaque formation was observed. Cell damage, which was apparent grossly, sometimes occurred when the dehydration time exceeded 1 day. It was noted that the dehydration time with which optimal plaque formation was obtained frequently coincided with, or preceded by only a few hours, the time at which slight cell damage first appeared. Use of a dehydration time greater than 2 days sometimes resulted in severe cell damage and a concomitant sharp reduction in plaque formation.

TABLE 1. *Effect of dehydration* of cell monolayers on infectivity of poliovirus RNA*

Period of dehydration of cell monolayers	Avg no. of plaques per bottle after dehydration of cell monolayers	
	With caps left on bottles	With caps removed from bottles
5 min	1	0
15 min	2	2
30 min	6	7
1 hr	5	12
3 hr	10	195
5 hr	8	90
7 hr	7	6†
10 hr	27	
20 hr	130	
32 hr	28†	
44 hr	37†	
58 hr	12†	
72 hr	11†	

* Cell monolayers were washed twice with isotonic saline and then allowed to drain (dehydrate) for various periods before inoculation with poliovirus RNA in isotonic diluent.

† Cell damage.

With the use of cell monolayers dehydrated with the caps left off the bottles, the plaque formation also increased with an increase in dehydration time, but in this case the plaque formation was increased with a much shorter period of dehydration (Table 1). The optimal plaque formation was obtained with cell monolayers dehydrated only 1 to 5 hr, and the degree of increase in plaque formation was of the same order as with cell monolayers dehydrated for 1 day with the caps on the bottles.

When dehydration of the cell monolayers was carried out with the caps left off the bottles, cell damage was encountered more frequently than when the caps were left on the bottles during dehydration. This increased cell damage was possibly due to excessive drying or progressively alkaline conditions, or perhaps both of these factors.

When dehydration of cell monolayers was carried out with the caps on the bottles at various temperatures for periods of 1, 6, 12, and 24 hr, little difference in plaque formation was obtained with cell monolayers dehydrated at 4, 26, and 36 C.

Inasmuch as a good plaque response and little or no apparent cell damage was obtained with cell

TABLE 2. *Plaque formation of poliovirus RNA on cell monolayers treated with various concentrations of ethyl alcohol[a] prior to inoculation*

Concn of ethyl alcohol used in treatment of cell monolayers	Avg no. of plaques per bottle
%	
None[b]	6
1	2
2.5	6
5	8
10	126
15	>500[c]
20	—[d]
25	—[d]
30	—[d]

[a] Cell monolayers were washed twice with isotonic saline and then treated with 0.2 ml of appropriate ethyl alcohol solution. After 1.5 hr were allowed for dehydration, the cell monolayers were inoculated with poliovirus RNA in isotonic diluent.

[b] Isotonic saline used for control.

[c] Confluent.

[d] Cell damage.

monolayers dehydrated at room temperature for 1 day with the caps on the bottles, this method was used in the following experiments.

An examination of the period allowed for adsorption of the poliovirus RNA onto dehydrated cell monolayers revealed that the greatest infectivity was obtained with an adsorption of approximately 2 to 5 min. When the adsorption time was prolonged for much longer than 10 min, a considerable loss in infectivity usually occurred. Little difference in infectivity was obtained when adsorption was carried out at 4, 26, and 36 C.

Studies were made concerning the washing of the cell monolayers prior to dehydration. With isotonic saline, which was the solution used in most of the experiments, the highest infectivity was obtained with cell monolayers washed one to three times. When the cell monolayers were washed 5 or 10 times, no further increase in plaque formation was obtained. When the cell monolayers were not washed at all, but the nutrient medium merely was poured off and dehydration was allowed to take place, little or no plaque formation occurred.

It was found that isotonic sucrose could replace the isotonic saline as the wash solution, and good plaque formation could be obtained with the dehydrated cell monolayers. The use of water as the wash solution caused total destruction of the cell monolayers. This destruction was not always observed at the time of washing of the cell monolayers but did appear at various times during the experiment.

In most of the experiments, the cell monolayers were 1 day old when the dehydration period was begun. When infectivity of RNA preparations was studied with cell monolayers aged 1 to 4 days prior to dehydration, little variation in plaque number was noted.

When RNA preparations were diluted in isotonic saline and then assayed on dehydrated cell monolayers, it was found that the plaque formation decreased in an irregular manner. It was noted, however, that a much higher plaque response was obtained with the first dilution made (1:5 or 1:10) than with the undiluted RNA (Ludwig and Smull, 1964).

In other experiments, a small volume of ethyl alcohol was applied to the cell monolayers prior to the beginning of the dehydration period. It was thought that if loss of water from the cell was a factor in increasing the efficiency of infection with poliovirus RNA, the use of a dehydrating agent might accelerate the drying process and thus the plaque response. Experimentation revealed that the plaque formation of the RNA was indeed greatly increased when assayed on cell monolayers treated with certain concentrations of ethyl alcohol prior to inoculation (Table 2). Concentrations of ethyl alcohol ranging from 10 to 20% were found to be the most effective ones for this purpose. When ethyl alcohol concentrations greater than 30% were used, even for short periods, severe cell damage usually was incurred.

With a fixed dehydration time, the plaque formation generally increased with an increase in ethyl alcohol concentration up to the limit of cell tolerance of the alcohol. The effect of the ethyl alcohol on increase in plaque formation was also readily demonstrated with the use of a fixed concentration of ethyl alcohol on cell monolayers for various periods of time. The plaque formation of the RNA on cell monolayers treated with 0.2 ml of 10 or 15% ethyl alcohol and then allowed to dehydrate for 1 to 1.5 hr prior to inoculation of the RNA was approximately equal to that obtained with cell monolayers dehydrated for 24 hr without ethyl alcohol treatment.

Additional experimentation revealed that it was possible to revert well-dehydrated cell monolayers to their original almost totally insusceptible state merely by moistening them with a few drops of isotonic saline immediately prior to inoculation of the RNA. This finding indicated that the RNA-cell interaction which resulted eventually in plaque formation was an immediate one; otherwise, the wetting of the cell monolayers with the inoculum would probably have prevented it from

taking place. This RNA-cell interaction was apparently also a stable one, as washing of the dehydrated cell monolayers immediately after inoculation of RNA resulted in no loss in plaque formation.

When isotonic sucrose or water was used for wetting of dehydrated cell monolayers, prior to inoculation of RNA, a complete lack of infectivity also resulted.

Experiments with poliovirus RNA applied in a basic protein-containing diluent. When poliovirus RNA in a histone-containing diluent was assayed as described earlier (Ludwig and Smull, 1963) on dehydrated cell monolayers, it was found that the plaque formation, like that of RNA in isotonic saline, increased with an increase in dehydration time, with optimal plaque formation obtained with cell monolayers dehydrated for approximately 4 to 24 hr. With histone-RNA preparations, the increase in plaque formation was noted with cell monolayers drained for only 10 min, in contrast to the prolonged dehydration time necessary before any appreciable increase in plaque formation with RNA in isotonic saline. With the use of appropriately dehydrated cell monolayers, the plaque formation of the histone-RNA preparations was approximately 10 times that obtained with undried cell monolayers.

Experiments with poliovirus RNA in hypertonic diluent. When poliovirus RNA in 1.0 or 1.25 M NaCl diluent was assayed on cell monolayers dehydrated for periods up to 2 days, it was found that the plaque formation was increased only slightly or not at all with the use of dehydrated cell monolayers. However, when RNA applied in diluents ranging from 0.25 to 0.75 M was assayed on cell monolayers dehydrated for 24 hr, a much greater plaque response was obtained than when assay was made on cell monolayers drained for 5 to 10 min.

Experiments with poliovirus. It was found that the infectivity of poliovirus, in contrast to that of the nucleic acid, was not altered when assayed, according to standard techniques, on cell monolayers dehydrated for periods up to 2 days.

Increased virus production by dehydrated cell monolayers. An interesting observation made during the experimental period was that under certain conditions the plaques formed on well-dehydrated cell monolayers were considerably larger than those formed on undried cell monolayers of the same age. The large plaques were formed when infection was initiated either by whole virus or by the nucleic acid, and occurred mainly on cell monolayers which were approximately 1 day old when the dehydration period was begun. The increase in plaque size was such that plaques formed on cell monolayers aged 1 day and then dehydrated for 1 day had a diameter approximately twice that of plaques formed on undried 2-day-old cell monolayers. When the cell monolayers were aged 2 or 3 days prior to a 24-hr dehydration period, the plaques formed were the same size or only slightly larger than plaques formed on undried cell monolayers of the same age.

TABLE 3. *Poliovirus production* in dehydrated and undried cell monolayers*

Incubation prior to harvest	Virus production (PFU/ml)	
	24-hr dehydrated cell monolayers	Undried cell monolayers
hr		
7	9×10^2	2.7×10^2
16	2.4×10^3	8×10^2
28	7.2×10^4	2.4×10^3
89	3.0×10^5	4.5×10^4

* Infection was initiated by whole virus.

It seemed logical to presume that a larger plaque size was indicative of an increased virus production in the dehydrated cell monolayers, and experiments were carried out to demonstrate such an increase. Inasmuch as poliovirus formed approximately the same number of plaques on dehydrated as on undried cell monolayers, it was used in preference to poliovirus RNA in these studies. Such experiments, as described in Materials and Methods, revealed that there was a considerable increase in virus production in the dehydrated cell monolayers as compared with undried ones (Table 3).

In one other experiment, dehydrated cell monolayers were moistened with isotonic saline just prior to inoculation with poliovirus. It was found that, with this treatment, the large plaques still formed and the number of plaques formed was the same as that on similarly treated undried cell monolayers.

At the present time, further efforts are being made to determine the reason for the increased virus production in dehydrated cell monolayers.

DISCUSSION

The infectivity of the isolated nucleic acid of poliovirus, like that of several other virus nucleic acids, is very low compared with that of the whole virus preparation.

This great loss in infectivity may be due to such factors as injury to the nucleic acid during the extraction process, to inability of the nucleic acid to attach properly to and enter host cells, or to the presence of RNA inhibitors in the nucleic

acid preparations or in the environment of the host cells. The possibility also exists that suitable methods for measuring the total infectivity of nucleic acid preparations are not available.

In general, the methods for increasing the infectivity of poliovirus RNA include a manipulation with the RNA (such as addition of a hypertonic solution, a poorly water-soluble substance, or a basic protein) after which the RNA is applied to cell monolayers, which often have been handled normally. In contrast, in the studies described here, the basic manipulation concerned the host cell, after which the RNA (in the same form obtained from the extraction process, except for dilution in isotonic saline) was applied.

The results obtained in this study strongly indicate that dehydration of the cell is a most important factor in increasing the efficiency of infection with poliovirus RNA. As a result of studies of infectious RNA in tissue culture with the use of solutions of varying tonicity, Ellem and Colter (1960, 1961) suggested that withdrawal of water from the cell was an important factor in increasing the efficiency of infection of the RNA. Thus, the data in the present study support the conclusion of these workers.

Assuming that dehydration of the cell does bring about an increase in efficiency of infection with RNA, there are several ways in which this might occur.

Ellem and Colter (1960) suggested that dehydration acts by causing loss of activity of RNA inhibitors. This would certainly seem to be a logical explanation. Several workers (Sprunt, Koenig, and Alexander, 1961; Norman and Veomett, 1961; Smull and Ludwig, 1962) have reported that an inactivator of infectious RNA can be found in the environment of cells in culture. In addition, it was shown that this cell-associated RNA inactivator can be inhibited by hypertonic solutions (Sprunt et al., 1961). RNA inhibitors, both inside and outside the cell, might be inactivated by a hypertonic environment brought about by dehydration.

The reversion of dehydrated cells to a nonsusceptible state by moistening them with isotonic saline, as described in the present study, might be explained on the basis of activation of RNA inhibitors by the isotonic saline. With a return of the cells to an isotonic state, the RNA inhibitors could again become active.

Another possible mode of action of dehydration would be through pinocytosis. Application of the RNA inoculum to the dehydrated cells might stimulate pinocytosis with subsequent engulfment of infectious RNA particles. In this regard, pinocytosis might cause an increase in infectivity of RNA with hypertonic solutions and basic proteins. Pinocytosis can be induced in amoebae with the use of certain salts and proteins (Chapman-Andresen and Prescott, 1956; Chapman-Andresen, 1958).

It is possible that dehydration causes damage to the cell and in this way brings about an increase in infectivity of the RNA. It was noted consistently in this study, in the experiments concerned with varying periods of dehydration, that optimal plaque formation occurred at the time, or just before, cell damage became grossly evident. Koch, Koenig, and Alexander (1960) reported optimal plaque formation of poliovirus RNA, applied in hypertonic diluent, on cell monolayers showing obvious cell damage.

The fact that the infectivity of poliovirus was not improved with the use of dehydrated cell monolayers is not surprising, as the virus apparently has a very effective means of its own of attaching to and entering cells. Any inactivation of RNA inhibitors by the dehydrated conditions would not affect the virus, because this infectious unit is not susceptible to their action anyway.

There are probably several mechanisms by which the efficiency of infection with RNA in tissue culture is increased, and it is likely that one or more of them function as a result of dehydration of the host cell. However, it is also probable that at least some of the mechanisms function through some means other than dehydration. It is difficult to explain improvement of plaque formation of poliovirus RNA by the method of Dubes and Klingler (1961) or the basic protein method on the basis of dehydration.

Even with the several methods now available for improving the efficiency of infection with poliovirus RNA in tissue culture, at best the infectivity is still only a small fraction of that of the whole virus. This indicates that some factor, such as a subtle injury to the RNA during extraction, or inadequacy of present methods of measuring the total infectivity of RNA preparations, may exist which is uninfluenced by the present methods of improvement.

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LITERATURE CITED

ALEXANDER, H. E., G. KOCH, I. M. MOUNTAIN, AND O. VAN DAMME. 1958. Infectivity of ribonucleic acid from poliovirus in human cell monolayers. J. Exp. Med. **108**:493–506.

BOEYÉ, A. 1959. Induction of a mutation in poliovirus by nitrous acid. Virology **9**:691–700.

CHAPMAN-ANDRESEN, C. 1958. Pinocytosis of inorganic salts by *Amoeba proteus* (*Chaos diffluens*). Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim. **31**:77–92.

CHAPMAN-ANDRESEN, C., AND D. M. PRESCOTT. 1956. Studies on pinocytosis in the amoebae *Chaos chaos* and *Amoeba proteus*. Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim. **30**:57–78.

DUBES, G. R., AND E. A. KLINGLER. 1961. Facilitation of infection of monkey cells with poliovirus "ribonucleic acid." Science **133**:99–100.

ELLEM, K. A. O., AND J. S. COLTER. 1960. The interaction of infectious ribonucleic acid with a mammalian cell line. I. Relationship between the osmotic pressure of the medium and the production of infectious centers. Virology **11**:434–443.

ELLEM, K. A. O., AND J. S. COLTER. 1961. The interaction of infectious ribonucleic acids with mammalian cells. III. Comparison of infection and RNA uptake in the Hela cell—polio and L cell—Mengo RNA systems. Virology **15**:113–126.

GIERER, A., AND G. SCHRAMM. 1956. Die Infektiosität der Nucleinsäure aus Tabakmosaikvirus. Z. Naturforsch. **11b**:138–142.

HOLLAND, J. J., B. H. HOYER, L. C. MCLAREN, AND J. T. SYVERTON. 1960. Enteroviral ribonucleic acid. I. Recovery from virus and assimilation by cells. J. Exp. Med. **112**:821–839.

HOLLAND, J. J., AND L. C. MCLAREN. 1959. Improved method for staining cell monolayers for virus plaque counts. J. Bacteriol. **78**:596–597.

KOCH, G., S. KOENIG, AND H. E. ALEXANDER. 1960. Quantitative studies on the infectivity of ribonucleic acid from partially purified and highly purified poliovirus preparations. Virology **10**:329–343.

LUDWIG, E. H., AND C. E. SMULL. 1963. Infectivity of histone-poliovirus ribonucleic acid preparations. J. Bacteriol. **85**:1334–1338.

LUDWIG, E. H., AND C. E. SMULL. 1964. Dilution of poliovirus ribonucleic acid as an important step in obtaining infectivity with histone. J. Bacteriol. **87**:964–965.

NORMAN, A., AND R. C. VEOMETT. 1961. Ribonuclease activity at the HeLa cell surface. Virology **14**:497–498.

SMULL, C. E., AND E. H. LUDWIG. 1962. Enhancement of the plaque-forming capacity of poliovirus ribonucleic acid with basic proteins. J. Bacteriol. **84**:1035–1040.

SMULL, C. E., M. F. MALLETTE, AND E. H. LUDWIG. 1961. The use of basic proteins to increase the infectivity of enterovirus ribonucleic acid. Biochem. Biophys. Res. Commun. **5**:247–249.

SPRUNT, K., S. KOENIG, AND H. E. ALEXANDER. 1961. Factors influencing the degree of infectivity of enterovirus ribonucleic acid (RNA). II. Role of host cell RNA inactivators in the low degree of infectivity of poliovirus RNA in low salt concentration. Virology **13**:135–138.