OBSERVATIONS CONCERNING A PERSISTING INFECTION OF HeLa CELLS WITH POLIOMYELITIS VIRUS*

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(Received for publication, July 14 1955)

In nature degrees of interaction between cells and viruses must occur and, depending upon the integration achieved, a variety of reactions result, which are the usual phenomena associated with virus infections (1). This is most apparent from considerations of comparative virology. Compare for example, the various combinations of influenza virus with the cells of the chorionic membrane (2), the allantois (3), with the muscle fibroblasts of the chick (4), of the monkey kidney (5), HeLa cells (6), and mouse brain (7). This range of host cells can support viral multiplication with or without marked cytopathogenic effect, induce infection and cytopathology without viral increase, or establish infection and multiplication without viral release. Recent findings suggest further that a single complex host-virus system could exhibit its multiple associated phenomena autonomously. Under certain metabolic influences, it is possible to observe the infection of HeLa cells by poliomyelitis virus with an accompanying cytopathogenic effect but without viral multiplication (8). This implies that a single host and virus are capable of varying degrees of integration. Indeed the capacity for limited degrees of integration may be a usual factor in the natural history of virus diseases, particulary when one observes them beyond the acute phase.

The present study is a long term observation of a viral infection in a tissue culture and bears upon this problem. The pertinent observation is that under suitable environmental circumstances, poliomyelitis virus can persist in a culture of HeLa cells while the cells are undergoing prolonged and extensive multiplication. The integration of three variables, susceptible cells, virus, and immune serum, to produce a stable host-virus relationship has been investigated.

Methods and Materials

Tissue Cultures.—All lines of HeLa cell employed were obtained directly from Dr. J. T. Syverton. The cultures were maintained with close approximation to the method described by Syverton, Scherer, and Elwood (9). Certain modifications, however, were made in the procedure and in the growth medium. Cultures were washed twice with maintenance solution

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

after the growth-supporting medium was removed and before the cells were dispersed with trypsin. This was done as routine on all transfers. Tube cultures of HeLa cells were used for most experiments and for viral titrations. The number of cells used to inoculate bottles or tubes was as described by the above authors.

Growth Medium.—The medium used to support cellular propagation was a fluid composed of human serum (40 per cent) of undetermined antibody content and balanced salt solution (60 per cent). No fraction of the old medium from a previous passage or chick embryo extract was employed, since these are not required for prolonged cultivation and only increase the chances of contamination. All cultures contained penicillin and streptomycin.

Maintenance Solution.—For certain purposes, cultures were maintained in a medium free of human serum. It was a balanced salt solution supplemented with amino acids, vitamins, and some carbohydrate intermediates. However, in the place of 10 per cent chicken serum (9), horse serum was employed.

Viruses.—The strains of poliomyelitis virus used were: Mahoney (Type I), MEF (Type II), Saukett (Type III).

Type-Specific Monkey Sera.—The three type-specific sera used were prepared in the laboratory of Dr. Wenner by inoculation of monkeys.

Viral Titration.—The amount of virus was estimated by determining the limiting dilution which would initiate infection in 50 per cent of the tube cultures of HeLa cells inoculated. Serial 10-fold dilutions of virus were prepared in maintenance solution and 0.1 ml. aliquots of each dilution were added to four tube cultures. The cultures were examined for 7 days for cytologic changes characteristic of infection. The dilutions recorded are final dilutions. Since all tube cultures contained 1 ml. of fluid, all titers are expressed as the number of tissue culture infectious doses ($TCID_{50}$) per ml. of sample. The 50 per cent endpoint was calculated by the method of Reed and Muench.

OBSERVATIONS AND EXPERIMENTS

Origin and Description of the R Line of HeLa Cells.—As a matter of routine, several lines of HeLa cells are maintained in the laboratory and subcultured separately. Originally all these lines of cells were obtained directly from the laboratory of Dr. Syverton. The observations which are reported in this study concern the last 31 passages of a particular line of cells, to be designated as R, which were recognized as being different from several other lines of HeLa cells. Superficial observation under low magnification of cultures of the R line growing in test tubes showed the cells to be somewhat elongated, and while growing in a well dispersed monolayer, they were sufficiently distant so that a confluent sheet was never produced. When supplied with a suitable human serum or pools of human serum, the cells multiplied at a rate comparable to other lines and could be subcultured repeatedly. The total viral increase produced in 31 serial passages was 1×10^{14}-fold. The multiplication occurring in each passage is recorded in Table I.

Requirement of Immune Human Serum.—When the data from individual passages shown in Table I were considered, it was clear that in some instances very poor cellular multiplication had occurred. Apparently the cultural conditions employed during these particular passages were unsuitable. This point was considered further and, in light of other observations, the several individual sera used in pools for cultivation were studied as the possible pertinent variable.

The various sera were tested first for their capacity to neutralize the three types of poliomyelitis virus using the standard neutralization test in tissue cultures of HeLa cells. Two groups of sera were separated: those with antibodies to all three types of poliomyelitis virus and those without antibodies to any type of poliomyelitis virus. A serum was considered negative only if at a dilution of 1–4 it would not neutralize 100 tissue culture infectious units of virus. Next the individual sera were tested for their capacity to support the multiplication of the R culture.

TABLE I

Extended Observation of Persisting Virus Infection

Passage Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Cell Multiplication	–	3	6	2	6	3	4	2	1	5	2	2	2	4	6	4	4	5	1	1	5	4	4	5	3	4	3	1	2	2	4
Cell Instability	–	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+
Virus Production	–	–	–	–	–	+	+	+	–	–	+	–	0	0	0	+	+	+	+	–	+	0	+	0	0	0	0	0	+	0	0

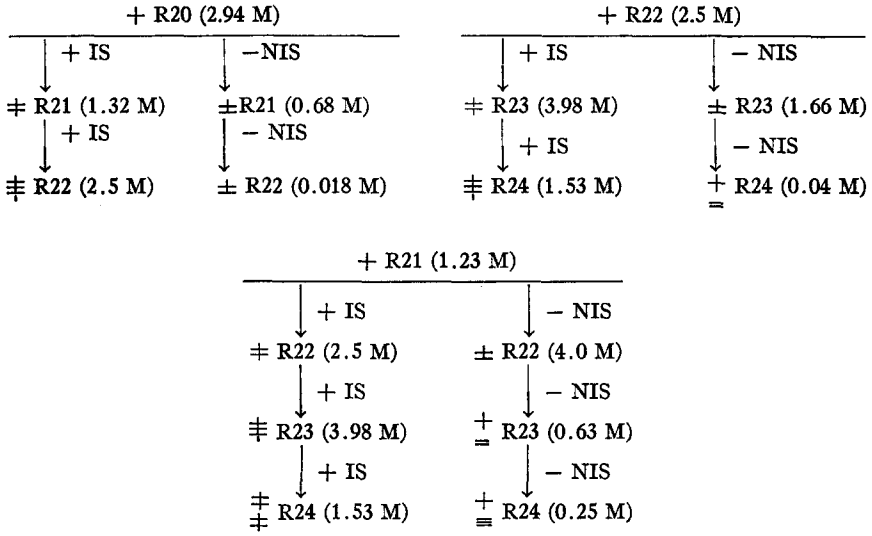
Cellular multiplication is expressed as the fold increase observed over the original inoculum which occurred by the seventh day of incubation. The + symbol under cell instability indicates an observation of cellular disintegration upon removal of immune serum. Under the heading of Virus Production, it indicates a successful attempt to isolate virus. The 0 symbol indicates negative observations, while the – indicates no attempt at observation was made.

These sera were tested upon several passages of the R line of cells. A particular passage of cells was chosen and subcultured in two bottles, under parallel conditions, using the serum free of antibody and that containing antibody. At the end of each subpassage the cultures were treated with trypsin and the cells counted. New bottles were seeded with 8×10^5 cells. If the recovery was less than 8×10^5, new cultures were seeded with as many cells as recovered. The results from three experiments in which passages R20, R21, and R22 were analyzed by subculturing are given in Fig. 1. It will be noted that in each case some multiplication occurred upon the first passage in the presence of nonimmune serum, but sustained multiplication and subcultivation were possible only with the immune serum. Apparently the R line of cells differs from other cultures of HeLa cells in that it requires for sustained growth, serum from individuals who have had previous experience with poliomyelitis virus. As yet a large survey of human sera with various combinations of types of antibody has not been made. However, with those tested the correlation holds.

Instability of the R Line in Presence of Maintenance Solution.—Cultures of HeLa cells prepared from standard lines of cells are quite stable for 7 days in the presence of maintenance solution. This medium is employed as routine to grow virus in the absence of human serum which commonly contains antibody. The human serum is, of course, an essential component of the growth-supporting medium used to produce the original cellular outgrowth. Early in the study of the R line, it was noted that certain morphological changes could be induced in well developed cultures by replacing the growth-supporting medium by maintenance solution.

Replicate cultures from each of the passages of the R line from the second to the twenty-sixth were tested for this instability in the presence of maintenance solution. Cultures in test tubes were examined under low magnification without staining. The morphologic changes seen varied somewhat from one passage to another, but all cultures except the 13th exhibited obvious instability. The changes consisted of clumping of cells, granulation, rounding and in many cases, but not all, the rounded cells escaped from the glass surface. The gross appearance of the culture was similar to a HeLa culture infected with poliomyelitis virus.

FIG. 1. Immune serum requirement for cultivation of R line of HeLa Cells.



The values in parentheses represent the number of millions of cells per culture at the end of the growing period. The inoculum was 8×10^5 cells per culture or as many cells as available if less than that value. The abbreviations IS and NIS stand for immune serum and non-immune serum. The number following R is the passage number of the R strain of HeLa cells.

To explain the variation in response of the several passages and to quantitate the visual impressions gained, subcultures of several passages were prepared and studied.

Four bottle cultures were seeded with 8×10^5 cells obtained from the 25th passage of the R line. Two cultures, +R26, were overlayed with growth medium containing immune human serum while the other two, −R26, received non-immune serum. After 7 days of incubation, one culture, the +R26, which received the immune serum and one culture of the −R26 receiving non-immune serum were treated with trypsin and the cells counted. The remaining +R26 and −R26 cultures were overlayed with maintenance solution, incubated 5 days and then treated with trypsin and the cells counted.

Before the replacement of the growth medium, the culture of +R26 had 2.71 million cells. After standing with maintenance solution, the count was 2.33 million. The corresponding values of the −R26 were 2.33 million and 0.44 million respectively. Similar results were obtained with the R27 and R28 passages. Cells which are injured are susceptible to the hydrolytic action of trypsin and are not visible in the hemocytometer used to enumerate cells. The differential count gives a measure of the number of the cells undergoing disintegration.

Apparently the stability of the R line in maintenance solution is related to the immunologic character of the human serum in which cells are immediately grown. The potential for instability, however, persists even when the cells have undergone 31 passages in the presence of protective serum. These data and those in the previous section suggest that the protective element of the immune serum may be carried with the cells through several cellular divisions. The endogenous protective element can support limited cellular division or survival in the absence of immune serum in the medium.

Viral Production Associated with Induced Cellular Disintegration.—Attempts to isolate an infectious agent from R cultures were prompted by the resemblance between the cytologic changes which occurred in those cultures and in those which can be produced by viral action.

The first attempts to isolate an infectious agent were made by inoculating a standard line of HeLa cells with the extracellular fluid from the R6 passage which had been induced to disintegrate by removal of immune serum. This procedure produced in the ordinary culture of HeLa cells a typical viral cytopathogenic effect. Further, the destructive principle could be subcultured repeatedly in standard lines of HeLa cells. Using the neutralization technique in tissue cultures of HeLa cells, it was possible to identify the agent with hyperimmune monkey serum as Type III poliomyelitis virus. The virus was then inoculated intracerebrally into a *rhesus* monkey. Subsequently, the animal developed paralysis. Microscopic examination of the monkey cord showed lesions typical of poliomyelitis. Cells from 22 of the 31 passages were tested for their potential to give rise to virus. From eleven passages isolations were made; however, attempts to isolate virus from eleven other passages failed. The particular cultures tested and the results obtained are recorded in Table I.

The infectious agent isolated from the 23rd passage was also studied and identified.

A bottle culture was prepared with an inoculum of 8×10^5 cells obtained from the 22nd passage of the R culture which had been carried as routine with immune human serum. The culture was incubated 7 days with growth medium containing non-immune serum. The growth medium was removed and maintenance solution was added. After 3 days of further incubation, deterioration of the culture was observed. The extracellular fluid was removed and titrated in standard HeLa cells and in kidney cells. The corresponding titers were $10^{5.5}$ and $10^{2.5}$. By the use of hyperimmune type-specific monkey serum, the virus was identified in tissue cultures of

HeLa cells as Type III poliomyelitis virus. To further identify the virus, mice were inoculated intraperitoneally with 0.5 ml. of the maintenance solution removed from the deteriorated R23 culture. After 2 weeks the mice were challenged intraspinally with an intraspinal variant of Type III poliomyelitis virus (Li-Leon). In the control groups challenged with 10^{-1} and 10^{-2} dilutions of virus, 8 of 8 and 8 of 9 animals became paralyzed. In the experimental group challenged with equal amounts of virus, the corresponding values were 7 of 8 and 3 of 9. The protective effect was also indicated by the slower rate at which the experimental animals became paralyzed. The harmonic means of the survival time in days for the control groups were 2.5 and 3.2, while those for the experimental groups were 4.8 and 21.3.

The degree of protection afforded by a single intraperitoneal inoculation was considered significant and further indicated that the isolate was Type III poliomyelitis virus.

However, upon intraspinal inoculation or intracerebral inoculation of mice, the material was non-infectious. Intracerebral inoculation of a *rhesus* monkey also failed to produce paralysis, and there was no antibody rise. A pool of this virus was prepared in a standard culture of HeLa cells. The same monkey received a course of vaccination with the virus of this pool. Subsequent testing of this animal's serum with three types of poliomyelitis virus (Mahoney, MEF., and Saukett strains) showed a specific rise to the Saukett strain.[1] The titer was 1 to 2400.

From these findings it is clear that only certain passages of the R line of cells give rise to poliomyelitis virus (Table I). This yielding of virus when it does occur is associated with instability of the culture in the absence of immune serum. Apparently, in the presence of immune human serum, the virus has persisted in some form from the sixth to the twenty-eighth passage even though it was not detectable with the techniques used in certain of the passages. The properties of the virus isolated from the 23rd passage are different from those of the virus isolated from the sixth passage. However, the serologic type remained fixed.

Failure to Isolate Virus from R Cultures.—From the data of Table I, it will be noted that attempts to isolate virus from a number of passages were unsuccessful. However, the cells of these same passages showed the typical instability in the absence of immune serum. Several of the cultures (R26, R27, R28) were those described in a previous section. They had been subcultured once with non-immune serum, treated with maintenance solution for several days, dispersed with trypsin, and counted. The growth medium which was removed, the maintenance solution, and the trypsin-treated cell debris were all tested with a variety of dilutions in stable HeLa cells cultures in order to isolate virus. High concentrations (10^{-1} dilutions) of the trypsin-digested material produced some rounding of cells but no toxicity could be transmitted by subculturing. The maintenance solution was also tested with negative results in cultures of kidney cells, in mice by intracerebral and intraspinal routes, and in a monkey by the intracerebral route.

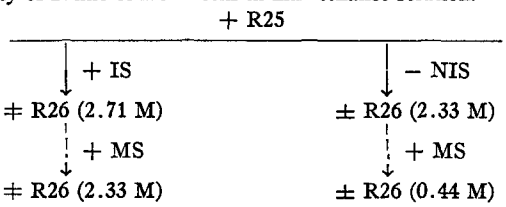
It will also be recalled that when disintegration of these R cultures, *i.e.* R26,

[1] The experiments with animals were performed in collaboration with Dr. Kenneth W. Cochran and will be reported in greater detail at a later time.

R27, R28 which did not yield virus, was induced the extent of disintegration was 80 per cent (Fig. 2). Since no infectious agent was liberated upon disintegration which would infect other susceptible cells or animals or the remaining 20 per cent of HeLa cells in the sensitive culture, it would appear that at least 80 per cent of the culture is composed of unstable cells requiring immune serum for survival and multiplication.

Attempts were also made to isolate virus without prolonged treatment with maintenance solution from a culture (passage 19) which could be induced to yield virus by the usual treatment with maintenance solution.

FIG. 2. Instability of R line of HeLa cells in maintenance solution.



Values in parentheses represent the number of millions of cells per culture at the end of the growing period or incubation period. The inoculum was 8×10^5 cells per culture. The abbreviations IS, NIS, and MS stand for immune serum, non-immune serum, and maintenance solution. The number following R is the passage number of the R strain of HeLa cells. The broken arrows represent further treatment of the culture without subcultivation.

The medium from this culture was removed; the cells attached to the glass wall were washed twice with 8 ml. volumes of maintenance solution. Then with the aid of a rubber policeman, the cells were scraped from the glass, suspended in maintenance solution, and homogenized in a glass Potter-Elvejhem homogenizer. The homogenate was examined microscopically and found to contain few whole cells. The preparation was then centrifuged lightly. The supernatant was supplemented with 20 per cent horse serum and the entire volume layered over the cells of a culture of stable HeLa cells. By this procedure involving no dilution, it was hoped that a single infectious unit of virus might be detected. After 7 days no evidence of cytopathogenic effect could be detected in the culture. The extracellular fluid of the test culture was then repassed to tube cultures and reexamined for 7 days with negative results.

Thus it was not possible to isolate virus from disrupted cells of the nineteenth passage, yet a replicate culture yielded virus upon prolonged incubation in the absence of human serum.

Intermittently, series of passages of the R culture failed to yield virus detectable by infectivity in a number of susceptible systems. These same cells retain the instability property of the R culture and the potentiality to produce virus upon prolonged subcultivation. At present, the factors which determine whether the induced cellular cytopathology will be accompanied by detectable infectious virus or not, have not been defined.

Rate of Viral Increase Following Induction of an R Culture.—In those series

of passages in which the cellular deterioration was accompanied by the appearance of virus, it was of particular interest to know something of the time relationships involved.

Six test tube cultures were prepared using cells from the 20th passage. Each tube was seeded with 25×10^3 cells and the culture allowed to grow in the presence of serum for 5 days. The six mature cultures (+R21) were then washed free of growing medium with maintenance solution, overlayed with the same medium, and incubated further. At various intervals, 0.1 ml. was removed from all tubes and pooled. The aliquots were replaced with fresh maintenance solution. The samples pooled from each time interval were titrated for virus in standard HeLa cultures. The resulting data are plotted in Fig. 3.

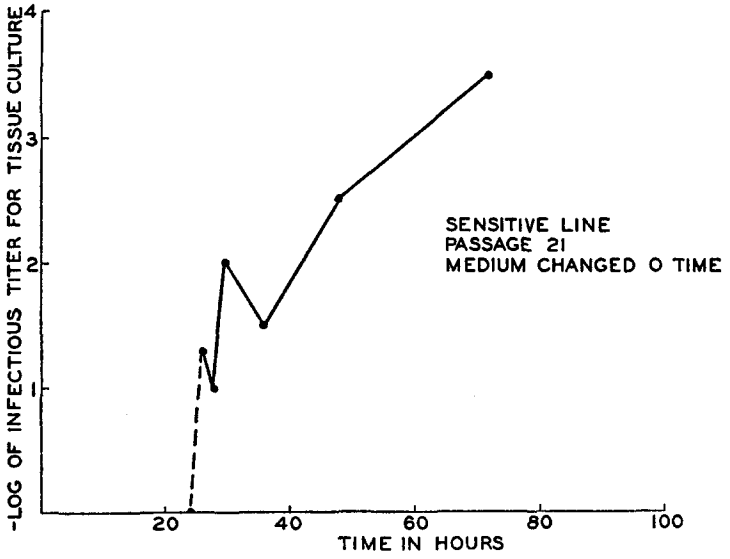


FIG. 3. Each point represents the value obtained by titration of a pool consisting of samples from six cultures. Samples were also taken at 4, 6, 8, 12, 16, and 22 hours; however, no virus could be detected in the aliquots taken.

During the first 24 hours, no virus could be detected by the inoculation of 0.1 ml. portions of undiluted fluid into HeLa cultures. The first appearance of virus occurred between the 24th and 26th hour. The titer increased slowly for the next 60 hours. Fluids obtained from a number of other passages which yielded virus were also titrated for virus. The titers ranged from $10^{-6.0}$ to $10^{-5.5}$ for the +R26 and +R28 to $10^{-2.5}$ for the +R19.

Establishment of a New Persisting Infection.—It was considered that if the factors uncovered in the study of the R culture were truly pertinent, it should be possible to establish a second persisting infection using selected human serum, a stable line of HeLa cells, and the virus isolated from the R culture.

A suspension of cells was prepared with trypsin from a stable line of HeLa cells. The trypsin was removed by centrifugation and the cells resuspended in balanced salt solution to give a concentration of 140×10^3 cells per ml. To 4.8 ml. of this suspension was added 0.1 ml. of a 10^{-1} dilution of tissue culture fluid containing virus isolated from the R-6 culture. Approximately $10^{4.0}$ tissue culture doses of virus were used. The mixture was transferred to a culture bottle and after 1 hour 3.2 ml. of immune human serum was added. The culture was incubated at 37°C. for 7 days and then subcultured. This line of cells was designated as the T line.

The *T* line has undergone 15 subpassages producing approximately $10^{9.0}$-fold cellular increase. Each passage showed the instability in maintenance solution characteristic of the R culture. From eleven passages (1, 2, 3, 4, 5, 6, 7, 10, 11, 12, 15) it was possible to isolate virus. As in the case of the R line, certain passages (8, 9, 13, 14) showed instability without yielding virus.

It has been noted from visual observations that few cells survive the initial addition of virus which is made for the establishment of new carrier lines. After the addition of virus, most cells are destroyed, and the culture with the persisting infection arises from the outgrowth of surviving cells.

DISCUSSION

The essential observations are that poliomyelitis virus could be isolated periodically from cultures of HeLa cells being repeatedly subcultured and that serum from persons who have had previous experience with the virus was an obligate requirement for survival of the system. The inability to detect virus in some passages also indicates that the virus does not persist in the R culture always in a state exhibiting the infectious property. What persists is rather the potentiality of the R culture to give rise to fully active virus.

These observations indicate that a modified type of cell continues to reproduce, transferring unto the daughter cells poliomyelitis virus whose destructive effect is suppressed in the presence of antibody in the nutrient medium. Under these conditions it is not possible to estimate whether the presence of virus contributes to resistance of the cells or whether it stimulates any antibody formation by them. It is of interest, however, that some degree of resistance appears to remain for one or two transfers in the absence of added antibody. But subsequently the cytolytic effect occurs as the infected cell is freed from inhibition.

It is possible to postulate a mechanism for the persistence of infection, which assumes that only a few cells in the culture were infected at any particular time. However, there are several experimental facts which suggest that the persistence of infection has its basis in a cellular phenomenon in contrast to a cultural or ecological one: first, the culture arises in the beginning from only a small portion of the original population of cells; second, the general appearance of the carrier culture differs from that of standard lines of HeLa cells; third, from many passages it was not possible to isolate virus; and fourth,

nearly 80 per cent of the cells in some passages can despoil themselves without producing demonstrable infectious virus.

Regardless of the mechanisms involved, the R cell requires the presence of immune serum for its continuation, and the persistence of viral antigen depends in turn upon the survival of the R cell and its progeny. Thus, indirectly, the persistence of the virus antigen is dependent also upon the presence of immune serum. In an *in vivo* system, the persistence of immunity should be favored by the ability of the virus to appear at times of declining immunity. The concept of a persisting antigen being a causal factor in lasting immunity is an old one. However, the idea that a state of immunity might abet the survival of virus rather than its elimination is intriguing. It suggests that the immune state represents a balanced host-parasite relation rather than only a refractory condition and brings to mind the persistence of an infectious agent following immunity against a disease. It has been pointed out that when a virus is shown to persist in the supposedly recovered individual, relapses of the infection may not uncommonly occur (10, 11). Whether they are usually preceded or accompanied by a decline in specific antibody is not established. But the association between antibody decline and relapses has been well demonstrated in certain infections such as malaria (12).

The system employed in the present studies provides an excellent model of the relapse or reactivation of virus which may well be applicable to conditions such as lymphocytic choriomeningitis, varicella, and herpes zoster, Brill's disease, psittacosis, or others.

SUMMARY

A culture of HeLa cells has been subjected to prolonged observation with the finding that periodically Type III poliomyelitis virus could be isolated from it. A requirement of the culture for survival was the presence in it of serum of certain individuals who had had previous experience with poliomyelitis virus. In the presence of serum containing no antibodies to poliomyelitis virus, the culture demonstrated spontaneous cytopathology. From certain series of passages virus could be isolated while attempts were unsuccessful from others also showing cellular disintegration. The conclusion is reached that the virus does not persist in the culture always in a state exhibiting the infectious property, rather what persists is the potentiality of the culture to give rise to fully active virus. The immune serum could inhibit the cytopathogenic effect of the virus without eliminating the infection.

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