MECHANISMS OF PERSISTENT AND MASKED INFECTIONS IN TISSUE CULTURE*

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It has been repeatedly observed that certain infected tissues that initially cannot be made to yield virus upon isolation will do so after the tissues have been subcultured *in vitro* (Rowe *et al.*, 1953, and Hull, Minner, and Smith, 1956). This cultivation *in vitro* subjects the cells to new environmental influences. The cells are transformed from a resting state to one of rapid proliferation; furthermore, they are removed from the humoral influences of the host. Once isolated, the viruses are apparently cytopathogenic to the progeny of the cells that formerly harbored them. If one disregards the possibility that the cultures were repeatedly contaminated after isolation, one must assume that the virus was present in the tissue in some nontransmissible or masked form. The factors involving the length of time the virus had persisted in the tissue and whether it had been undergoing some restricted multiplication present questions of considerable interest.

Several years ago we made the observation that poliovirus could be repeatedly isolated from subcultures of HeLa cells in which prolonged and extensive cellular multiplication was occurring (Ackermann and Kurtz, 1955). In this experimental system there was strong evidence that the virus not only persisted, but to some degree replicated. More recently, this type of observation *in vitro* has been extended to the adenoviruses (Ginsberg and Boyer, 1956).

The aim of this presentation is to consider what relation these *in vitro* systems bear to the phenomenon described by Rowe and Hull and their associates, on the one hand, and to the problem of recurring infections and persisting immunity, on the other.

The first efforts at explanation should be in terms of the known framework and principles of the single infectious sequence, and one should be cautious in postulating new principles and laws. From this viewpoint it may be worth while to note that there is a nontransmissible phase in the normal infectious sequence of animal viruses. Further, if the normal sequence of development is interrupted by the use of metabolic antagonists after infection has been initiated, as has been done with influenza virus (Ackermann and Maassab, 1955), or by the use of deficient medium as applied to psittacosis (Morgan, 1956), the infected cell can remain in a state of virostasis for long periods, even for days. The period of virostasis may be terminated upon change in the environment, and the infectious sequence will then continue and will yield virus. In the case in which virostasis was induced with an inhibitor of protein synthesis, viral development still continued in this noninfectious stage.

The fact that virus in the noninfectious state is quite durable, perhaps more so than when in the extracellular infectious form, is further illustrated by recombination experiments in which irradiated influenza virus was found to react

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with host cells, to persist for as long as 4 days, and then to undergo recombination when active virus was added (Baron and Jensen, 1955).

These findings are illuminating with regard to the persistence of virus without reference to the principle of lysogeny that is used to describe persistence and controlled multiplication of an infectious agent without cytopathology.

In regard to the masking of viral activity and the consequent difficulties in the detection of virus, particularly in the presence of inhibitors, it should be noted that neutral mixtures of virus and antibody that will not produce an extending or consuming infection in a HeLa culture (that is, not an overt cytopathogenic effect) and in the presence of which the cells propagate readily can be made to yield virus by a number of other procedures. Details of these procedures will be considered later. It suffices to stress the point that, while a virus may exist under certain circumstances in a nonpathogenic state, this phenomenon may be quite unrelated to our inability to demonstrate viral activity in certain preparations.

Properties of the Carrier State

Origin. The essential part of the observation *in vitro* considered here has already been recorded in the literature (Ackermann and Kurtz, 1955). In summary, when a culture of HeLa cells is exposed for 1 hour to a large amount of poliovirus and is subsequently washed and treated with immune serum, most of the cells show the degenerative changes typical of virus action. A small portion (about 6 per cent) of the original population survives and retains the capacity to multiply. Such cells have been subcultured over 30 times, multiplying by 10^{18}. For reasons that will become obvious later, these cell populations have been designated as carrier cultures.

Morphology and respiration. The cultures derived from these surviving cells have been found to differ in several interesting aspects from standard growths. Their distinctive morphology is illustrated in the accompanying series of photomicrographs. The standard cell line at low magnification (FIGURE 1) shows characteristic polygonal cells oriented in a close-fitting pattern. It will be noted in the corresponding culture of carrier cells (FIGURE 2) that individual cells are elongated and grow in a dispersed pattern. At higher magnification (FIGURE 3) it is clearly seen that the cells are not only elongated, but that the cytoplasm is small as compared to the nucleus. Whatever processes they have extend from the polar extremes. These details are in clear contrast to those of the standard culture (FIGURE 4).

A 30-per cent lower oxygen consumption per cell, as measured in the usual growth medium, corresponds with this reduction in cytoplasm. As is the case with standard cultures, the respiration of the carrier cell is stimulated by the addition of glutamine. However, at maximum stimulation the rate of respiration is 30 per cent lower than that of the standard cell (TABLE 1).

Superinfection of the carrier culture. The differences in metabolism and morphology between these cell types led us to study the reaction of the carrier cells to superinfection with other viruses. The carrier cultures were stabilized with immune serum of a type corresponding to that of the virus used to produce the

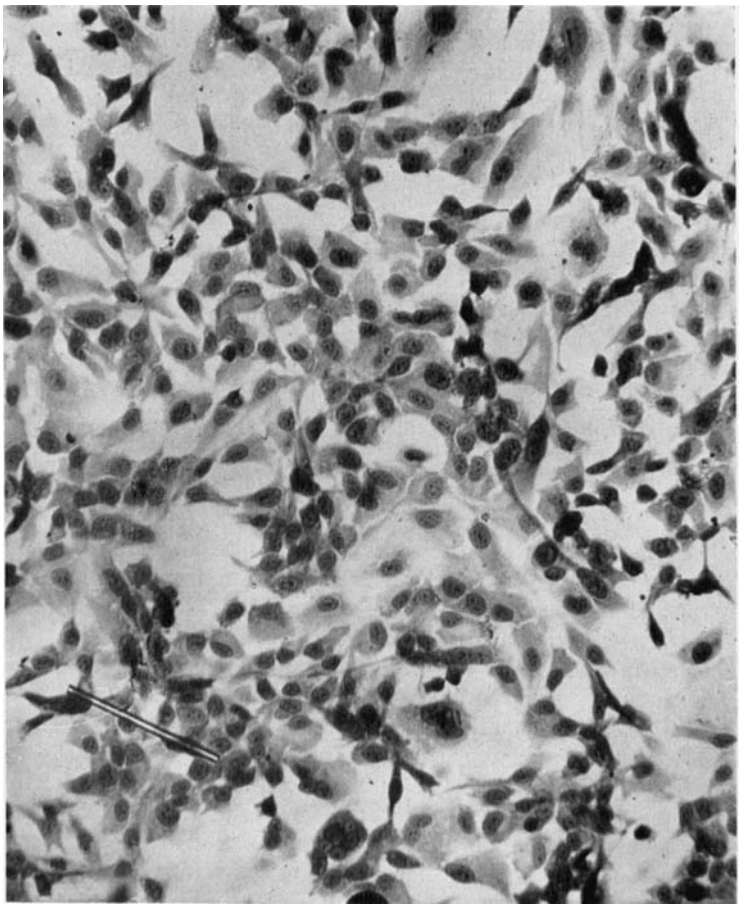

FIGURE 1. Standard culture of HeLa cells grown on a cover slip; stained by Giemsa's method. About ×50.

carrier culture; varying amounts of poliovirus of a second type were then added. When infection was initiated with any type of poliovirus or Coxsackie virus, the visible cytopathology developed more slowly in carrier than in standard cells.

This subjective visual impression was confirmed by counting the cells remaining attached to the glass wall at some suitable interval of time after infection (TABLE 2). For example, when the 2 cultures were infected with 10^2 TCD_{50} of Type 2 virus, 76 per cent of the cells remained in the carrier line at 48 hours, while 4 per cent remained in the standard culture. However, it should be noted that the cultures were susceptible to the same degree in that both could be infected with equally small quantities of virus despite the fact that the rate of deterioration was different.

Rate of viral development after superinfection. The slow rate of cellular

destruction apparently results from a slow rate of viral multiplication in the carrier line. When parallel cultures of standard and carrier lines were infected with the same amount of virus and when the growth curve in the culture was then followed, there was a lag of 16 to 18 hours in the time required for titers of virus in the carrier line to reach values comparable to those of the standard culture. Once viral increase begins, however, it appears to proceed at the same rate in each culture (FIGURE 5).

Action of immune serum upon infected carrier cells. The lag phase in the carrier culture was further studied by determining the sensitivity of the cells to immune serum after exposure to virus. This was effected by exposing carrier and standard lines to a large inoculum of virus for 1 hour and then removing the virus and overlaying with immune serum. The extent of cellular destruc-

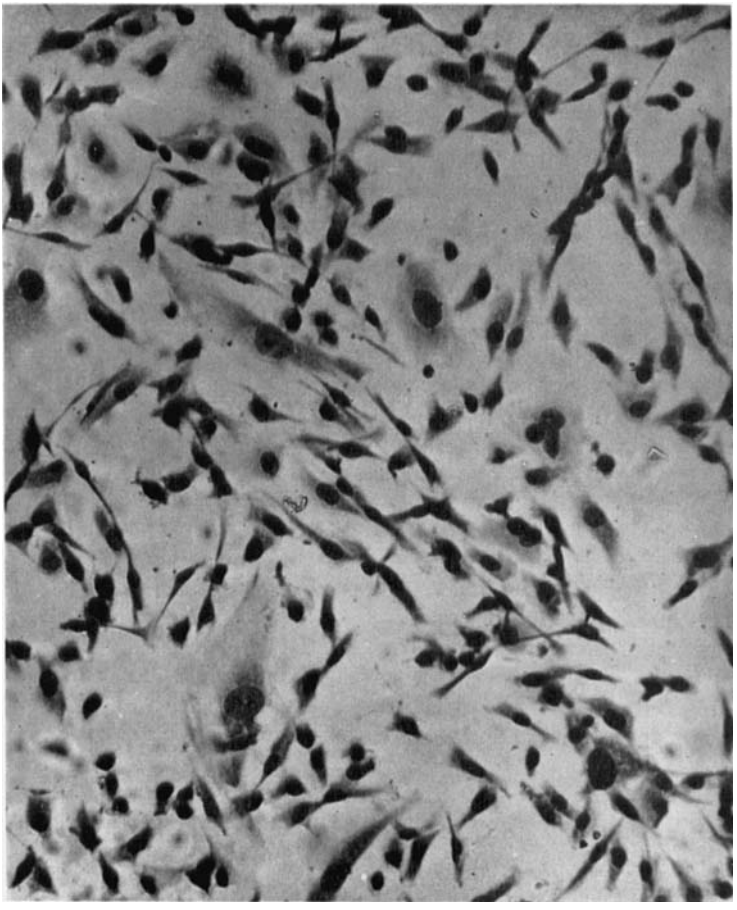

FIGURE 2. Culture of HeLa cells subjected to poliovirus Type 1 and subcultured in immune sera 15 times. This culture was grown on a cover slip and stained by Giemsa's method under conditions closely paralleling those of the culture in FIGURE 1. About ×50.

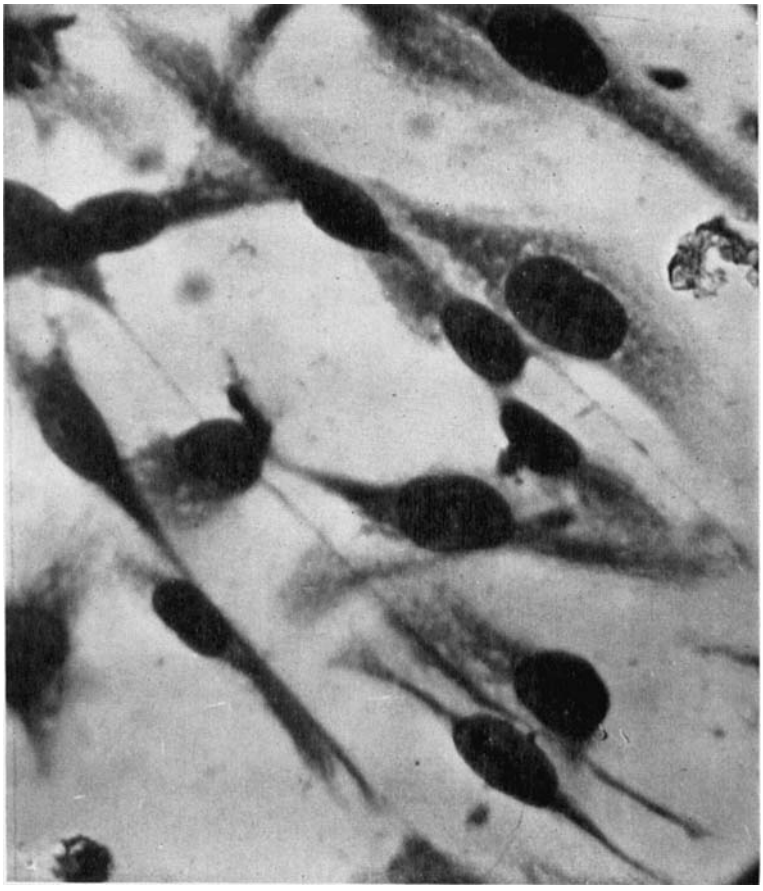

FIGURE 3. Higher magnification of the culture shown in FIGURE 2. About ×500.

tion was then observed. The evidence from such experiments indicates a remarkable difference in the response of the 2 lines. For example, in the standard HeLa culture, 67 per cent of the cells were infected and destroyed, while a parallel treatment of the carrier line destroyed only 3 per cent. Clearly, after exposure to virus, either the carrier cell is more sensitive to the action of immune serum or the early stages of infection are unusually prolonged.

Response of carrier culture to removal of antibody. As yet, it has not been determined whether these strange cells arise by selection of variants originally present in the cell population at the time the line was established or whether they arise by a process of induction as a result of intimate association with virus. However, if the growth-supporting medium containing antibody is removed, the culture will disintegrate spontaneously and, quite often, but not in every instance, virus can be isolated (Ackermann and Kurtz, 1955). The virus isolated is of the same type originally used to produce the culture. When the mainte-

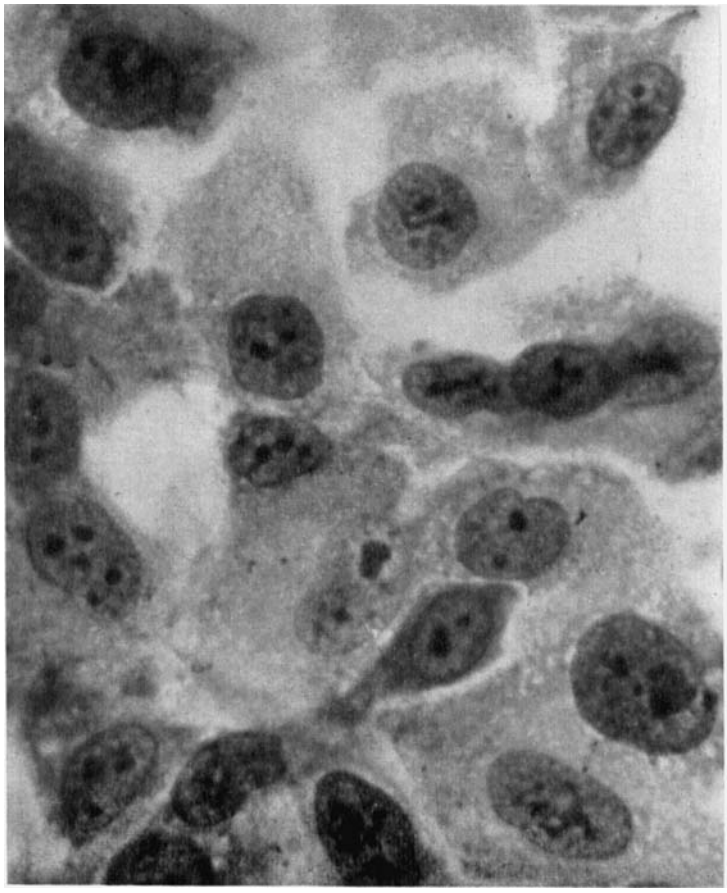

FIGURE 4. Higher magnification of the standard culture of HeLa cells shown in FIGURE 1; stained by Giemsa's method. About ×500.

nance solution in which disintegration occurs is supplemented with specific immune monkey serum, the culture is stabilized. While some multiplication of cells can be obtained with human serum free of antibody, this is effective for only one or two passages. Apparently the carrier state is not alone the result of an intrinsic property of the individual cell. There must be a function of immune serum in the system, even though it is mediated through special properties of the cell.

Action of Immune Serum

Without eliminating the virus completely, the essential action of immune serum must be either to restrict transmission of infection from cell to cell or to alter the developmental pattern of viruses in infected cells so as to allow continued cellular multiplication. The problem can be reduced to a determination

TABLE 1
RESPIRATION OF STANDARD AND CARRIER LINES OF HELA CELLS*

Supplement	Experiment number	O_2 uptake (μl. per 24 hours per 1,000,000 cells) 1/24 hrs./10^6 cells	
		Standard culture	Carrier culture
None	1	88.7	N.D.†
	2	78.3	47.9
	3	88.0	67.0
	4	81.0	59.0
	Average	84.0	57.9 (69%)
Glutamine 0.3 mg./ml.	1	103.5	N.D.†
	2	103.0	63.0
	3	101.8	70.7
	4	N.D.†	67.1
	Average	102.8 (100%)	66.9 (65%)

* HeLa cells from standard and carrier cultures were suspended from monolayers by the use of trypsin. The number of cells was determined with a hemocytometer, and oxygen consumption was measured in the Warburg apparatus over an 18-hour period. The cells were suspended in the usual growth medium composed of 60 per cent balanced salt solution and 40 per cent human serum.
† Not done.

as to whether every cell in the carrier culture simultaneously possesses the potential to give rise to virus at some time. An unequivocal answer cannot be adduced from the present data, but it may be worth while to consider what data are available and how they may influence our speculation.

While it is not known if all cells in the carrier culture are infected, it is clear that nearly all of them show distinctive morphology and that at least a fraction of the population is producing virus while the culture is undergoing net cellular increase in the presence of immune serum. The fact of survival and detection of virus in the growth medium of carrier cultures was established from the considerations discussed below.

TABLE 2
RATE OF DEVELOPMENT OF VIRAL CYTOPATHOLOGY IN STANDARD AND CARRIER LINES OF HELA CELLS*

Dilution of superinfecting virus		Cellular survival Control = 100	
		Standard culture	Carrier Type 1
Type 1	10^{-1}†	0.0	0.1
	10^{-4}	5.5	76.3
	10^{-5}	39.5	114.0
Type 2	10^{-3}‡	1.0	12.5
	10^{-4}	4.4	76.0
	10^{-5}	12.1	85.0

* In the studies of superinfection with Type 2 poliovirus the cultures were stabilized with Type 1 hyperimmune monkey serum. When Type 1 virus was used, the experiment was terminated before appreciable spontaneous disintegration of carrier cultures began.
† Forty-eight hours after virus addition.
‡ One hundred and twenty hours after virus addition.

Recovery of virus from neutral mixtures. If a mixture of virus and antibody is prepared in certain proportions and then layered over a culture of HeLa cells, it will not establish an extending or consuming viral infection and will not produce visible cultural degeneration even with the passage of time. Furthermore, proliferation of the host cell will occur in the presence of the mixture. Such preparations will be referred to as neutral mixtures. When a culture is exposed for several hours to such a neutral mixture, later washed repeatedly with maintenance solution, and then incubated further, the culture will degenerate and produce virus. In some experiments the supernatant fluid was removed; the culture was then washed thoroughly with a solution containing immune serum in order to remove the neutral mixture; finally, the culture was washed once more, this time with maintenance solution to remove the remaining immune serum. In this manner, possible dissociation of the neutral mixture was avoided. However, the same results were obtained as heretofore. In the latter experiments the phenomenon appears to be different from the infection that would follow the reactivation of antigen-antibody unions by simple dilution, and it implies the formation of some cell-virus complex prior to reduction of the antibody concentration.

These findings are consistent with much evidence in the literature indicating that cell-virus-antibody complexes can exist. Viral antibody can be adsorbed

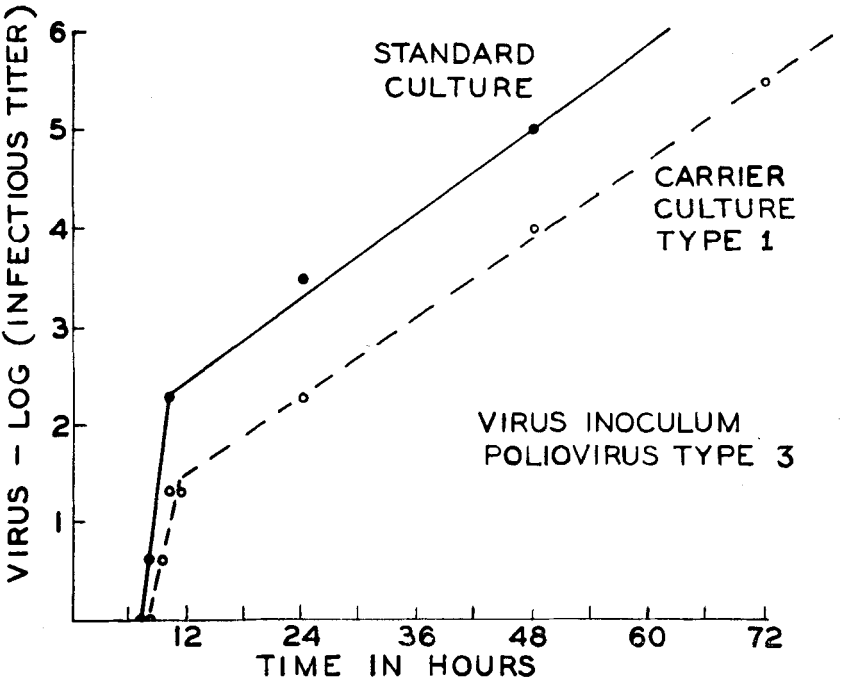

FIGURE 5. Rate of virus multiplication in standard and carrier cultures of HeLa cells. Parallel cultures of standard HeLa and carrier cells (Type 1) were prepared in bottles. The monolayers were overlaid with maintenance solution containing hyperimmune Type 1 monkey serum, and each layer was infected with approximately 10 TCD_{50} of Type 3 poliovirus. The supernatant fluid was sampled at varying intervals and titrated for virus in tissue cultures.

selectively by red blood cells to which influenza virus is irreversibly bound (Jensen and Francis, 1953), and there is also very strong evidence that viral antibody will react with complexes of chorioallantoic cells and virus (Ishida and Ackermann, 1956). Further, if phages are first neutralized with antibody, they will still bind to bacteria. It should be noted that virus in combination with antibody is not destroyed, and that its activity can be regenerated by displacement with inactivated virus, as shown by Hultin and McKee (1952), by Krech (1955) and, most recently, by Dulbecco (1956). Although the point is not fully clarified, it seems that under certain circumstances the complex may be dissociated by simple dilution. Many preparations of virus appear inhomogeneous, not only in their reaction with formalin and heat, but also antibody. There is some evidence to show that it may be possible to recover, from neutral mixtures, virus that does not react with antibody (Dulbecco, 1956). This is apparently not a genotypic variation but, rather, one due to the state of aggregation or physical form. The concept of a fraction of virus resistant to antibody and that of a reversible virus-antibody complex are not mutually exclusive.

Growth media of carrier cultures. The growth media of carrier cultures behave as neutral mixtures and, in many instances, they yield virus by exposure to susceptible cells, a process followed by the removal of excess antibody. The existence of virus in the medium was also demonstrated by preparing and inoculating the appropriate dilution into susceptible cultures. This is of some interest since these neutral mixtures had been standing over the carrier cells for several days, and one might have expected that any virus refractory to antibody would be adsorbed from the mixture by these cells, as was done later by the cells of the standard culture.

Thus, it becomes clear that either free virus or virus-antibody complex was present in the growth medium and could have produced some sort of infection of standard HeLa cells. From this it follows that at least a fraction of the cells in the carrier culture were producing virus while there was a net cellular increase. A generalized infection becomes manifest only when the cultures are freed of antibody. This is due either to a more widespread degeneration of a cell-virus-antibody complex in the presence of a reduced antibody concentration or to the generalized spread of an infection that formerly spread only by virtue of a small antibody-uncombined fraction of the viral yield.

Viral transmission in the presence of antibody. Significant to the problem is the question whether an infection can be maintained and transmitted at a low level through the medium in a culture of cells while the cells are retained under a neutral mixture. This is a difficult question to resolve for, while virus can be detected in neutral mixtures as described above, it is impossible quantitatively to measure virus in the presence of antibody by infectivity determinations. The problem was approached experimentally in the following manner:

A culture was prepared with a standard-size inoculum of HeLa cells and supplied with a medium composed of a neutral mixture of antibody and poliovirus. In 1 week the culture had grown into a complete monolayer of cells. The supernatant fluid was removed, and the cells were layered with maintenance solu-

tion. Upon further incubation the culture showed the degenerative changes characteristic of poliovirus, and a virus was isolated. The supernatant fluid was diluted with an equal volume of fresh medium and was used to grow a new culture of HeLa cells started with a standard inoculum. The procedure was repeated several times. Thus, by passing the culture medium (with some dilution and some additional immune serum) over fresh cells and testing it for virus in each passage, it was possible to learn if the isolable virus in the neutral mixture was a fixed residue that was being diluted away or whether it was being maintained by virus yielded by the new cells retained under the neutral mixture. Later passages were also carried in bottles that contained no cells. While these experiments are not conclusive, it has been clearly possible, in one series, to demonstrate virus in the supernatant fluid in 5 consecutive passages. In control passages containing no cells, the virus was lost by dilution and thermal inactivation after 2 passages.

Comment. The experiments with neutral mixtures of antibody and virus provide at least one mechanism to explain how virus may be restricted in its action by immune serum and yet not be eliminated thereby. If an infection is restricted to a fraction of the population of a culture, this fraction must remain constant if the infection is to persist. Since the culture is multiplying, the number of infected cells must also increase. At any given time, t, one would expect the number of virus-producing centers, I_t, to be related to the original number I_o in the following manner:

$$I_t = I_o P^{t/c}$$

where P is the mean effective fraction of the viral yield per cell that can function in the presence of antibody, and c is the time required for 1 cycle of infection. The rate of increase of the supporting cells in the culture would be related to the time for binary fission, to the number of viable cells, and to the magnitude of the fraction used to support the infection.

While the disintegration of carrier cultures in the absence of antibody without the production of virus remains difficult to explain, the morphological traits of carrier cells and the resistance to superinfection are the types of variation that one might expect to select by the procedure that produced the carrier lines of cells.

Regardless of the mechanisms involved, there still remains the primary observation that the action of immune serum upon an infected culture is not to eliminate the virus but, rather, to contribute to its survival. If, in the *intact* animal, such mechanisms are operative, the present observations provide an illustration at the cellular level of how resistance may develop in the presence of a stable host-parasite relationship.

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