STUDIES ON PERSISTENT INFECTIONS OF TISSUE CULTURES*

III. Some Quantitative Aspects of Host Cell-Virus Interactions

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The first report of this series (1) was concerned with the demonstration of persistent, inapparent infections in MCN or Lung-To cultures with Newcastle disease, mumps, or 6-6 viruses and an analysis of the effects of various manipulations upon the carried viruses, such as addition of antisera to the media or cloning of the cells. As a result of persistent infection the cultures were shown to acquire resistance to unrelated, cytopathogenic agents, such as the virus of vesicular stomatitis (VSV). The nature of this resistance has been examined in the second report (2) and the observed facts fulfill the criteria for viral interference. In the present study efforts were made to obtain data on the quantitative aspects of the host cell-virus interrelationships with respect to (*a*) the number of virus-producing cells in persistently infected cultures; (*b*) the number of infectious units present in virus-producing cells at any given time; and (*c*) the rate of propagation of virus and of development of interference to VSV upon transfer of a few infected cells from carrier to normal MCN cultures. The data obtained permit a tentative conclusion as to the mechanism which permits both cells and virus to persist simultaneously in cultures for indefinite periods of time.

Materials and Methods

All pertinent technics have been described or referred to in the preceding papers (1, 2).

EXPERIMENTAL

The Number of Virus-Producing Cells in Persistently Infected Cultures

The data presented in the first paper of this series imply that the vast majority of the cells in MCN or Lung-To cultures, persistently infected with

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Newcastle disease, mumps, or 6-6 viruses, are affected by the presence of these agents. The growth rate of the cells is reduced; the aerobic glycolysis is increased (3); and the cultures are highly resistant to certain cytopathogenic viruses. This, as will be shown in this section, does not denote that practically all cells are engaged in the reproduction of the carried viruses. It has been pointed out previously (1), that the amount of virus found in persistently infected cultures is always small in relation to the number of available cells. Furthermore, most of the virus is associated with the cells and usually only one and not more than 10 per cent of the agents is found free in the media. Thus the virus titers determined in the whole cultures essentially reflect the number of infected cells, which, in turn, seem to represent only a fraction of the total population. In order to obtain more accurate information on this point a number of different approaches were used.

Titration of Cells from Persistently Infected Cultures in Chick Embryos, HeLa, and MCN Cells.—These experiments were carried out in the following manner:

Washed cell suspensions were prepared from MCN_{NDV} cultures. The cells were counted in quadruplicate, and decreasing numbers were assayed for the presence of virus (*a*) by direct tests; *i.e.*, allantoic inoculation of chick embryos or inoculation into HeLa cultures. The allantoic fluids of the chick embryos were tested after 72 hours of incubation for viral hemagglutinins. The HeLa cultures were observed for 5 days for the appearance of characteristic cytolytic lesions of NDV; (*b*) by indirect tests; *i.e.*, the inoculation of MCN cultures. These were incubated for 2 to 3 weeks and the presence of NDV in individual cultures was then established either by subinoculation of chick embryos or HeLa cells or by determination of their resistance to 100 to 1000 TCD_{50} or VSV. From the data so obtained the number of cells required to infect 50 per cent of the host systems was calculated according to the method of Reed and Muench.

Results of a representative experiment are shown in Table I. It is seen that the various methods employed were of similar sensitivity except for the direct inoculation of HeLa cultures with MCN_{NDV} cell suspensions. In this instance 76 cells were needed to infect half the cultures. With the other methods from 25 to 33 cells were required. The latter variations were considered insignificant. In other experiments with MCN_{NDV} cultures, employing the direct injection of chick embryos or the indirect assay in MCN cultures the number of cells required for infection of 50 per cent of the hosts with few exceptions fell between 19 and 85. The direct titrations in HeLa cells always gave higher values in that between 76 and 300 cells were needed for infection of half the cultures. In occasional experiments the number of cells for 50 per cent infection of chick embryos was as large as 350 and inoculation of MCN cultures yielded positive results in only a fraction of the tubes to which 1000 MCN_{NDV} cells had been added. Fig. 1 summarizes graphically the results of three comparable experiments which were obtained by (*a*) direct injection of chick embryos and (*b*) by inoculation of MCN cultures followed in 2 to 3

weeks by sub-inoculation of chick embryos or challenge with VSV. As can be seen, the incidence of successful transmission of NDV infection was directly proportional to the number of MCN_{NDV} cells inoculated and no significant differences became apparent between the three methods of assay. All the points obtained by the various technics seem to fall onto a relatively narrow band with a slope of about 1.0. The spread of the points observed is accounted for by variations in the number of virus producing cells in different MCN_{NDV} cultures and by the inaccuracies inherent in the technics employed.

TABLE I

Number of MCN_{NDV} Cells Required to Transmit Infection to Chick Embryos, HeLa and MCN Cultures

No of MCN_{NDV} cells transferred	Chick embryos	HeLa cultures	MCN cultures			
			After 14 days		After 21 days	
			Subinoculation of chick embryos*	Resistance to VSV	Subinoculation of chick embryos*	Resistance to VSV
1×10^4	10/10‡	10/10§	10/10‡	10/10‖		
1×10^3	10/10	10/10	10/10	10/10		
1×10^2	9/10	6/10	9/10	9/10	7/10	7/10
5×10^1	7/10	2/10	8/10	14/20	8/10	16/20
2.5×10^1	4/10	0/10	3/10	9/20	5/10	10/20
1.25×10^1	1/10	1/10	2/10	5/40	1/10	2/20
0.63×10^1	0/10	0/10	1/10	3/40	1/10	1/20
No. of cells/EID_{50} or TCD_{50}¶	33	76	33	25	30	29

* Medium and cells from individual MCN cultures were inoculated into 2 to 3 chick embryos each.

‡ 10 out of 10 embryos contained hemagglutinins in the allantoic fluid.

§ 10 out of 10 HeLa cultures showed characteristic lytic lesions.

‖ 10 out of 10 MCN cultures resisted challenge with 1000 TCD_{50} of VSV.

¶ Calculated according to Reed and Muench.

The results show that in the persistently infected cultures only occasional cells are capable of transmitting infection to any one of the host systems studied. Of additional importance are the facts that (*a*) the incidence of successful transmissions of infection to MCN cultures by transfer of MCN_{NDV} cells did not increase with a prolongation of the incubation period from 2 to 3 weeks (Table I), or even to 1 month in other experiments; and (*b*) the final results in MCN cultures were indistinguishable from those obtained upon direct inoculation of chick embryos. These facts denote that only those cells which were infectious at the start of these experiments were capable of transmitting infection. In other words, no evidence was uncovered which would

suggest activation of virus in initially non-infectious cells during 3 to 4 week periods following transfer to MCN cultures. This interpretation was corroborated by the fact that transfer of intact or sonically disintegrated cells gave the same results (see below). A few experiments with $MCN_{6\text{-}6}$ and MCN_{Mp8} cells gave essentially similar results. The number of virus-producing cells in the former being, as a rule, greater than in the MCN_{NDV} cultures in accordance with the higher virus titers generally found in the $MCN_{6\text{-}6}$ preparations (1).

FIG. 1. Correlation of results obtained by inoculation of counted MCN_{NDV} cells into (a) chick embryos or (b) into MCN cultures followed in 2 to 3 weeks by subinoculation of chick embryos or by challenge with VSV.

The Number of Cells Entering into Virus Production on Transfer of MCN_{NDV} Cells to MCN Cultures.—The above experiments were carried out with MCN_{NDV} cells derived from the persistently infected stock cultures. It was of interest to determine whether MCN cultures, inoculated with a few MCN_{NDV} cells, after appropriate incubation periods again would reveal only a few infectious cells, or, whether under these conditions, a higher proportion of cells would enter into virus production. The experiment summarized in Table II shows that the former alternative is correct. In the first part of the experiment 10^3 MCN_{NDV} cells equivalent to 25 EID_{50} were inoculated into each of 20 MCN tubes. After 2 weeks of incubation 10 of the cultures

tested were solidly resistant to 1000 TCD_{50} of VSV. An additional 4 cultures were titrated at this time for NDV in chick embryos and $10^{4.6}$ EID_{50} were found per tube. The cells of the remaining 6 cultures were suspended by trypsinization, counted, and decreasing numbers were then seeded onto fresh MCN cultures. After an incubation period of 2 weeks these various groups were challenged with 1000 TCD_{50} of VSV. The results were in general agreement with those recorded above, in that 31 cells had to be transferred to induce resistance in 50 per cent of the cultures.

TABLE II

Number of Cells Entering into Virus Production in MCN Cultures after Transfer of MCN_{NDV} Cells

Part I	Part II		
Transfer of 1×10^3 MCN_{NDV} cells (25 EID_{50}) to MCN cultures and incubation for 2 wks.	Transfer of cells from Part I (2 wks. after transfer of MCN_{NDV} cells) to fresh MCN cultures.		
	No. of cells transferred	No. of EID_{50} transferred	Resistance to 1000 TCD_{50} of VSV 2 weeks after transfer
Resistance to VSV: 10/10	1×10^3	32	40/40
No. of EID_{50}/culture: 3×10^4	1×10^2	3.2	37/40
	5×10^1	1.6	33/40
	2.5×10^1	0.8	12/40
	1.25×10^1	0.4	7/40
	None	0	0/40
	No. of cells per TCD_{50}		31

Plaque Assays of Cell Suspensions from Persistently Infected Cultures.—Confirmation of the above results was sought by plating of cells from carrier cultures onto monolayers of chick embryo fibroblasts or HeLa cells in order to determine the number of plaques formed. The results were in essential agreement with the above data in so far as only a limited number of cells could be proven to be infected. Considerable variations were encountered in early tests, which were ascribed to the possibility that the seeded cells had not been fixed to the monolayers by the time the agar overlay was applied. Thus, the cells plated might have been trapped in the agar. This difficulty was overcome by prolongation of the interval between seeding of the cells and application of the overlay to 2 or more hours. The results of representative experiments are shown in Table III. It can be seen that between 43 and 105 MCN_{NDV} cells were required to yield one plaque on chick embryo fibroblasts, whereas 10 to 18 MCN_{6-6} cells gave one plaque on HeLa monolayers. The greater incidence of virus-producing cells in MCN_{6-6} as compared to MCN_{NDV} cultures has been noted also with the other technics described above.

Adsorption of Red Cells onto Cells in Persistently Infected Cultures.—It has been shown recently by Vogel and Shelokov (4) that tissue culture cells infected with influenza viruses adsorb red cells. This technic was applied to persistently infected MCN cultures.

TABLE III

Plaque Formation by MCN_{NDV} and $MCN_{6\text{-}6}$ Cells on Monolayers of Chick Embryo Fibroblasts or HeLa Cells

Experiment No.	Cells	No. of cells seeded	No. of plaques*	No. of cells per plaque
1	MCN_{NDV}‡	1×10^3	23	43
		1×10^2	2	50
2	MCN_{NDV} 1	1×10^3	18	56
	2	2×10^3	33	61
	3	2×10^3	43	46.5
	4	1.75×10^3	24	73
3	MCN_{NDV}	4×10^3	38	105
		2×10^3	24	83
		1×10^3	12	83
4	$MCN_{6\text{-}6}$§	1×10^3	59	17
		5×10^2	28	18
		2.5×10^2	21	12
		1.25×10^2	8	15
5	$MCN_{6\text{-}6}$	2.5×10^2	24	10
		1.25×10^2	12	10
		0.62×10^2	5	12.5

* Average of 3 to 4 plates each.
‡ On chick embryo fibroblasts.
§ On HeLa cells.

Guinea pig or chicken red cells were added to test tube cultures of uninfected or persistently infected MCN or Lung-To cells from which the media had been removed. The red cell suspensions (1 ml. 1 per cent) were permitted to stay on the cell sheets for 10 to 60 minutes at refrigerator temperature. Thereafter the cultures were washed 4 to 6 times and observed microscopically for adherence of erythrocytes to cells.

No hemadsorption was noted in any of the uninfected cultures. On the other hand, in MCN_{NDV}, $MCN_{6\text{-}6}$ and MCN_{Mps} cultures occasional cells were observed which were covered by red cells. Addition of immune serum prior to that of erythrocytes prevented specifically hemadsorption; *e.g.*, an anti-NDV serum inhibited adsorption in MCN_{NDV} but not in $MCN_{6\text{-}6}$ or MCN_{Mps} cultures. As has been pointed out previously (1), the media and disintegrated

cell suspensions of persistently infected cultures failed to agglutinate chicken, guinea pig, sheep, or human erythrocytes *in vitro*, the concentrations of virus present being too low to be detectable by the hemagglutination test. In the present experiments mere inspection revealed that the percentage of cells adsorbing red cells was obviously very much smaller than the fraction of cells which can generally be proven to be infected by the tests described above. Several possible explanations may be offered: (*a*) in handling of the cultures red cells were removed by the washing procedure; (*b*) the erythrocytes eluted before the observations could be made; and (*c*) only a small proportion of the infected cells contain sufficient virus material on their surfaces to permit recognizable hemadsorption.

The Number of Infectious Units Present in Infectious Cells

Efforts were made to determine the number of infectious units present in the infected cells. In earlier experiments MCN_{NDV} and MCN_{6-6} cell suspensions were subjected to 3 to 4 cycles of freezing at $-20°C.$ and thawing at room temperature or of alternate submersion in a dry ice—alcohol and a 37°C. water bath. In either case, the virus titers before and after treatment were of the same order. However, it was noted that the MCN cells were highly resistant to disruption by this procedure and a considerable percentage remained actually viable and cultures could be raised from them. Since these findings cast doubt on the validity of these experiments, the cells in subsequent tests were disrupted in the treatment vessel of a sonic oscillator (Raytheon 9000 kc). No intact cells could be discerned after 3 to 10 minutes of exposure.

Suspensions of MCN_{NDV} or MCN_{6-6} cells, containing between 250 and 7680 cells per ml., were exposed in 25 ml. volumes to sonic vibration for 5 to 30 minutes. Aliquots of the treated as well as untreated materials were then diluted in 2-fold steps and assayed for virus activity by one of the following methods: (*a*) allantoic inoculation of chick embryos; (*b*) inoculation of MCN cultures and challenge with VSV after 1, 2, and 3 weeks of incubation; and (*c*) plating on monolayers of chick embryo fibroblasts or HeLa cells.

The results of all 3 methods were similar in that sonically disrupted cells gave the same virus titers as the intact cell suspensions. If anything, sonic vibration rather decreased the virus titer in some of the experiments. Table IV summarizes two representative experiments employing the plaque technic of assay. The data indicate that the cells in persistently infected cultures, which can be proven to produce virus, contain probably not more than one infectious unit at any given time. This would imply that the virus particles are produced one at a time, and, as soon as completed, they are released from the cells. Exposure of NDV-infected allantoic fluid to sonic vibration for as long as 2 hours revealed no loss in titer.

The Development of Persistent Infection and Resistance to VSV

Further studies were concerned with the spread of virus and the induction of interference with VSV following infection of MCN cultures. Since evidence has been obtained (1) that NDV after being carried in MCN cells had undergone certain changes, as compared to the parent chick embryo-adapted strain, it was essential to determine the efficiency of the 2 lines of virus in establishing persistent infection and resistance to VSV.

TABLE IV

Plaque Counts Obtained with MCN_{NDV} and MCN_{6-6} Cells before and after Sonic Disruption

Cells	No. of cells seeded	Number of plaques			
		Intact cells		Disrupted cells	
		Range	Average	Range	Average
MCN_{NDV}*	2×10^3	13–42	24	10–34	21
	1×10^3	5–18	12	5–14	10
	0.5×10^3	2–16	8	5–17	9
	0.25×10^3	2–6	3	1–12	3
MCN_{6-6}‡	2.5×10^2	18–26	24	17–19	18
	1.25×10^2	9–14	12	9–10	10
	0.62×10^2	4–6	5	4–5	5
	0.31×10^2	2–3	2.7	1–4	2

* Chick embryo fibroblast monolayers; 8 plates per group.

‡ HeLa monolayers; 4 plates per group.

Adequate numbers of MCN cultures were inoculated either with decreasing quantities of allantoic fluids infected with the Victoria strain of NDV or decreasing numbers of MCN_{NDV} cells. The EID_{50} present in the inocula ranged from <1 to >1000. After 2 and 3 weeks of incubation presence or absence of NDV was determined in individual cultures by inoculation of the media into 3 to 4 chick embryos each and by challenge of the refed cell cultures with 1000 TCD_{50} of VSV. Between 20 and 40 cultures were used for each group.

The results of this experiment are summarized in Fig. 2. The number of EID_{50} inoculated is plotted against the percentage of cultures giving positive results. The MCN_{NDV} cell suspensions produced infection in 100 per cent of the cultures if they contained 10 or more EID_{50}. The percentage of takes declined sharply as the number of EID_{50} in the inocula decreased, but even with 0.2 EID_{50} a few cultures became infected. There was no significant difference in results when the incubation period extended over 2 or 3 weeks, or when chick embryos were sub-inoculated or resistance to VSV was tested. The chick embryo-adapted virus, on the other hand, required over 100 EID_{50} to infect all MCN cultures and with 50 EID_{50} only 25 per cent became infected or acquired resistance to VSV in 3 weeks of incubation. Furthermore,

the presence of virus in the cultures, as determined by inoculation of embryos, was not as yet necessarily evident from the results of challenge with VSV. Because of these differences between the NDV lines, further studies were carried out mainly with MCN-adapted NDV.

In the next experiment to be presented graded numbers of MCN_{NDV} cells were transferred to MCN cultures and the increase in virus was determined at intervals as well as the development of interference.

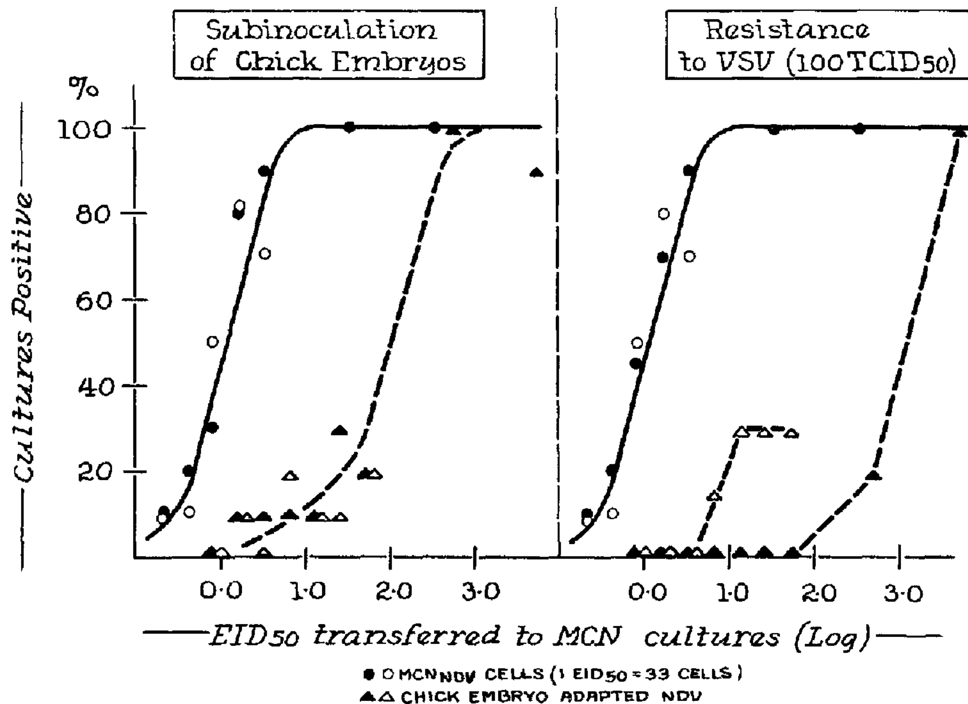

FIG. 2. The incidence of infection of MCN cultures 2 to 3 weeks following transfer of decreasing numbers of MCN_{NDV} cells or decreasing dilutions of chick embryo-adapted virus as measured by subinoculation of chick embryos or resistance to VSV.

Sufficient numbers of MCN cultures were inoculated with 25,000, 2500, or 250 MCN_{NDV} cells, representing 1500, 150, and 15 EID_{50}, respectively. Five tubes of each series were removed immediately after transfer of the cells and again at various days thereafter. These cultures were stored at −20°C. until plaque assays were made on monolayers of chick embryo fibroblasts. Titrations were made also in chick embryos. Other cultures were challenged for resistance to VSV.

It is seen in Fig. 3 that the infection progressed for several days at linear rates with slopes of about 1.0. Only the results of plaque assays are presented Those of chick embryo titrations were in principal agreement. After the con-

centration of virus had reached about 10^4 plaque-forming units (pfu), the titers continued to rise but at a markedly reduced rate. These late increases were roughly correlated to the increase in cells during the later incubation period. The proportion of virus-producing cells among the total transferred was unusually high in this experiment so that maximal infectivity levels and full resistance to VSV were attained in 3 days, the time of first challenge of interference. The same type of experiment carried out with NDV, derived

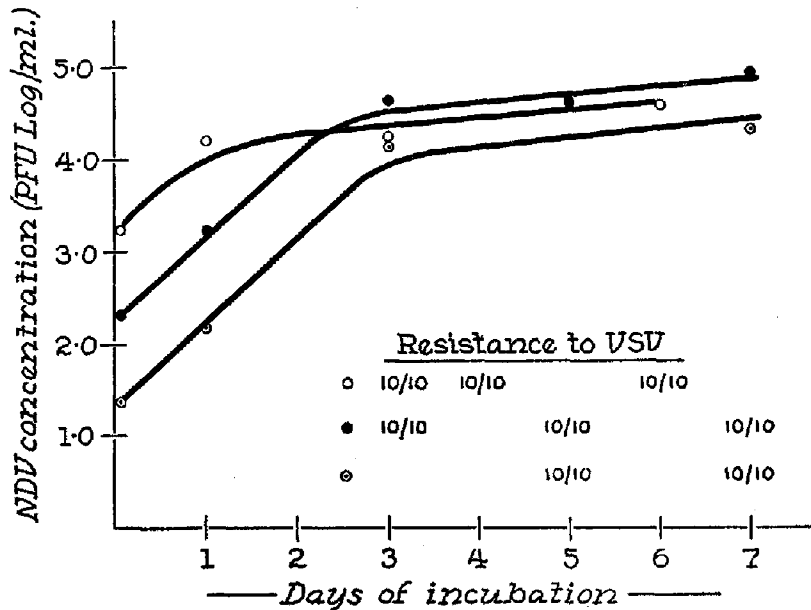

FIG. 3. Growth curve of NDV following transfer of 25,000, 2500, or 250 MCN_{NDV} cells (1500, 150, or 15 EID_{50} of NDV).

from MCN_{NDV} cultures by one allantoic passage, gave similar results. When chick-embryo-adapted virus was used, the NDV titers remained low for several days before they gradually started to increase and, even after 2 weeks of incubation, they had not as yet attained the levels obtained on transfer of MCN_{NDV} cells within 3 days.

Since in the above experiment interference was established by the time of first challenge another test was set up to determine the degree of resistance at shorter intervals following transfer of MCN_{NDV} cells.

Sets of adequate numbers of MCN cultures were inoculated with 10,000, 1000, or 100 MCN_{NDV} cells, or 250, 25, and 2.5 EID_{50} of NDV, respectively. On each of the ensuing 4 days, as well as after 1 and 2 weeks, 10 cultures of each group were challenged with 1000 TCD_{50} of VSV. In addition, 10 previously uninoculated MCN cultures were used for control of the

challenge virus and the VSV inoculum used was titrated to assure proper dosage. The cultures were read daily for 5 days. In most instances resistance was either complete (no VSV lesions) or entirely absent (4+ lesions). In a few cultures cytopathic effects were noted varying in degree from ± to 3+. For the recording of results any tubes with ± or 1+ lesions were considered resistant, those with more extensive destruction were counted as susceptible. In order to include partial effects in the evaluation of results the total lesion score was devised; *i.e.*, the sum of lesions observed in the 10 tubes of each group. Maximal destruction in all cultures thus is recorded as 40.

As can be seen in Table V, with transfer of 10,000 MCN_{NDV} cells or about 250 EID_{50} to MCN cultures, no resistance whatever was noted in 2 days but it was complete in 3. With one-tenth the number of cells, evidence of some

TABLE V

Development of Resistance to VSV after Transfer of Varying Numbers of MCN_{NDV} Cells to MCN Cultures

No. of MCN_{NDV} cells transferred	No. of EID_{50} transferred	Resistance of MCN cultures to VSV (10^3 TCD_{50})											
		Days after transfer of MCN_{NDV} cells											
		1		2		3		4		7		14	
		No. resistant	Lesion score*	No. resistant	Lesion score	No. resistant	Lesion score	No. resistant	Lesion score	No. resistant	Lesion score	No. resistant	Lesion score
1×10^4	250	0/10‡	40	0/10	40	10/10	0	10/10	0	10/10	0	10/10	0
1×10^3	25	0/10	40	0/10	40	1/10	31	9/10	5	10/10	0	10/10	0
1×10^2	2.5	0/10	40	0/10	40	0/10	40	3/10	29	3/10	26	4/10	24
0	0	0/10	40	0/10	40	0/10	40	0/10	40	0/10	40	0/10	40

* Sum of VSV lesions observed in 10 MCN cultures.

‡ Number of cultures protected/number challenged.

protection was apparent by the 3rd day, it was marked by the 4th, and complete by the 7th day, the next interval tested. Transfer of 100 MCN_{NDV} cells, or 2.5 EID_{50} protected only 3 or 4 out of the 10 cultures challenged each between the 4th and 14th day. The MCN cultures were originally seeded with 10^5 cells. With a generation time of about 48 hours, the number of cells by the 7th day was probably of the order of 10^6 (1). After this period no significant increases in cell populations were expected. The available data (2) indicate that at least one EID_{50} of NDV is required per cell to produce resistance to VSV. These considerations imply that the EID_{50} injected with the MCN_{NDV} cells into MCN cultures must have multiplied to the extent that at least 10^6 NDV particles became available during the period of observation. Yet, titrations for NDV in chick embryos or on monolayers of chick embryo fibroblasts never revealed concentrations of virus of that order.

DISCUSSION

It has been shown that once MCN or Lung-To cultures have been inoculated with Newcastle disease (NDV), mumps, or 6-6 viruses, they remain infected for indefinite periods of time, as long as they are properly maintained (1). The viruses persist at relatively low titers without causing recognizable cellular destruction. The presence of the agents is evident, however, from reduced cellular growth rates (1), increased aerobic glycolysis (3), and resistance of the cells to vesicular stomatitis (VSV) and certain other cytopathogenic viruses (1). The latter was shown to be an example of viral interference (2). In spite of these marked changes in behavior of the persistently infected cultures only one cell in 10 to >100 could be proven to yield infectious virus, depending upon the agent employed and possibly the conditions of the cultures used. This was demonstrated by (*a*) direct titrations of cell suspensions in chick embryos or HeLa cultures; (*b*) by transfer of decreasing numbers of cells to uninfected MCN cultures, followed after suitable incubation periods by subinoculation of chick embryos or HeLa cells, or by challenge with VSV as test for development of interference; (*c*) by plaque counts obtained on plating of counted cells onto monolayers of chick embryo fibroblasts or HeLa cells; and (*d*) by the Vogel-Shelokov technic (4) of adsorption of erythrocytes onto cells in persistently infected cultures. The fraction of virus-producing cells varied to some extent from test to test, with the virus employed and the method of assay. No evidence was obtained that the other cells (not producing virus) yielded non-infectious hemagglutinins or viral complement-fixing antigens (1) as observed on infection of MCN and several other cell lines with certain strains of influenza virus (5–7). Furthermore, these cells do not start to produce infectious virus at a later stage. On transfer of limited numbers of MCN_{NDV} cells to MCN cultures only those cells proven to yield virus at the time of inoculation were able to transmit the infection because (*a*) the incidence of successful transmissions was the same whether tested in 1 or 4 weeks; and (*b*) the same number of cultures became infected whether intact or sonically disintegrated cells were transferred. The latter point, indicating that each virus-producing cell contains at any given time not more than one infectious virus unit will be discussed later.

The above results reveal differences in susceptibility among MCN cells to NDV and the other myxoviruses employed. These differences could not be ascribed to genetic inhomogeneity of the cell population. As was shown previously (1), none of the cloned lines of MCN or MCN_{NDV} cells were destroyed and all became persistently infected by NDV. None were initially resistant to VSV but all lost their susceptibility when persistent NDV infection was established. In the absence of detectable genetic differences it must be assumed that only a few cells in the total population are in an appropriate physiological or nutritional state at the time of exposure to permit virus reproduction. These

may be referred to as "receptive" cells. The others, termed "refractory," at the time of contact with NDV or the other myxoviruses are incapable of supporting multiplication but are rendered resistant to VSV and the adsorbed NDV particles escape detection. The ratios between "receptive" and "refractory" cells are evident from the infectivity assays discussed above and range correspondingly from 1 to 10 to 1 to >100 depending upon the virus and possibly the cultural conditions.

Evidence has been obtained which suggests a selection of NDV particles during persistent infection of MCN cultures in that the agent emerging differs from the parent, chick embryo-adapted virus with respect to plaque morphology (1). This change in the virus is also evident in increased efficiency of establishing persistent, non-cytopathic infection and resistance to VSV in MCN cultures. This difference may be explained by the assumption that only a fraction of the egg-adapted NDV particles are capable of inducing limited viral reproduction following adsorption onto "receptive" cells. The spread of the infection throughout the inoculated cultures will therefore be slow and stable persistent infection and solid interference with VSV is thus achieved only with considerable delay. In contrast, MCN-adapted NDV, in form of MCN_{NDV} cells or first allantoic passage seeds derived therefrom, induces virus replication in presumably all receptive cells to which it becomes attached. The resulting progeny will be adsorbed mainly again onto "refractory" cells, which thereby acquire resistance to VSV, and only a few of the virus particles will enter "receptive" cells, thus perpetuating the infectious process. This cycling continues until all available "receptive" cells have been infected, and all "refractory" cells have been rendered resistant to VSV. The rate of increase in NDV in the inoculated cultures is initially logarithmic with a doubling every 6 to 8 hours. The rise in NDV in fact represents essentially the increases in virus-producing cells since, according to previous observations, most of the virus is associated with the cells and usually 1, and at most 10 per cent of it is found free in the media (1). Depending upon the number of MCN_{NDV} cells or of EID_{50} inoculated into MCN cultures, the logarithmic phase of increase in virus-producing cells may persist for 1 to 4 days; *i.e.*, up to the time when in the order of 10^4 such cells are present in the cultures. At that stage solid resistance to VSV is established. Thereafter the virus-producing cells increase at a markedly reduced rate in proportion to the growth of the cell population.

A number of problems exist with regard to the interpretation of the data just presented, which will be discussed now. Intact and sonically disintegrated cell suspensions from persistently infected cultures yielded the same numbers of plaques on monolayers of chick embryo fibroblasts or HeLa cells. It is thus evident that each virus-producing cell contains at any given time not more than about one infectious unit. This denotes that the virus progeny does not build up within the receptive cells but that virus particles as soon as completed,

are released from the cells. Such a mechanism of virus reproduction has been suggested also for influenza virus in the entoderm of the chick embryo allantois (7) or for equine encephalomyelitis virus in tissue culture (8). The rates of production of virus would appear to be low considering the twofold increase in virus-producing cells every 6 to 8 hours following transfer of small numbers of MCN_{NDV} cells to MCN cultures. However, by the time all available "receptive" cells (in the order of 10^4) have been infected, the total population, amounting to about 10^6 cells in the types of cultures used, has been rendered resistant to VSV. Since the equivalent of 10^6 EID_{50} of inactivated NDV are required to induce solid interference (2), it is evident that up to 100 times the number of virus particles must have been produced as were determined by the infectivity assays.

The course of events in the establishment of persistent infection thus far discussed entails an entirely extracellular spread of virus. The addition of anti-viral sera to the media should be effective therefore in curing the cultures of their infection. As was shown previously (1), antibodies reduced readily the extent of infection and interference but rarely achieved complete cures. The possibilities must be entertained that virus-producing cells may divide with the appearance of virus-producing daughter cells or that spread of infection may be attained on occasion by close contact between 2 cells. There is no question that the "refractory" cells in which interference has been induced, continue to divide, otherwise the persistently infected cultures would soon be lost. In experiments on interference with ultraviolet-inactivated NDV (2) it was shown in addition that the daughter cells were at least in part susceptible to VSV as long as the non-adsorbed interfering agent was removed from the cultures by washing or neutralized by antibody. Under conditions of persistent infection the daughter cells have the opportunity to adsorb immediately NDV from the medium. If they are "receptive," they serve to perpetuate the infectious process, if refractory, they are rendered resistant to VSV. That the daughter cells are at least in part also susceptible to VSV has been inferred from the growth curve experiments presented previously (2) in which it was shown that in challenged MCN_{NDV} cultures NDV and VSV compete for the new cells and the latter ultimately gains the upper hand.

From the data at hand and their interpretations as discussed, a scheme can be devised which seems to offer a satisfactory explanation for the establishment of persistent infection in the system under study and the simultaneous maintenance of both virus and cells in one culture. This scheme is summarized in Fig. 4 for infection of MCN cultures with MCN-adapted NDV. It is based upon the following assumptions which conform closely to the data presented: (*a*) the cell population at the start is 2×10^5 and doubles every 48 hours; (*b*) the number of cells capable of producing virus at all times is 1 in 50; (*c*) each virus-producing cell yields on the average 100 virus particles in 6 hours; (*d*) of

these 100 particles 2 absorb onto "receptive" cells, which then produce more virus. The other 98 attach to "refractory" cells, thus escaping direct detection but inducing interference with VSV; and (*e*) each infectious unit of NDV determined equals one infectious cell, the few free virus particles being disregarded. In order to achieve infection of all cultures, 5 virus-producing cells are transferred among 250 derived from a carried line. In a 4 day experimental period

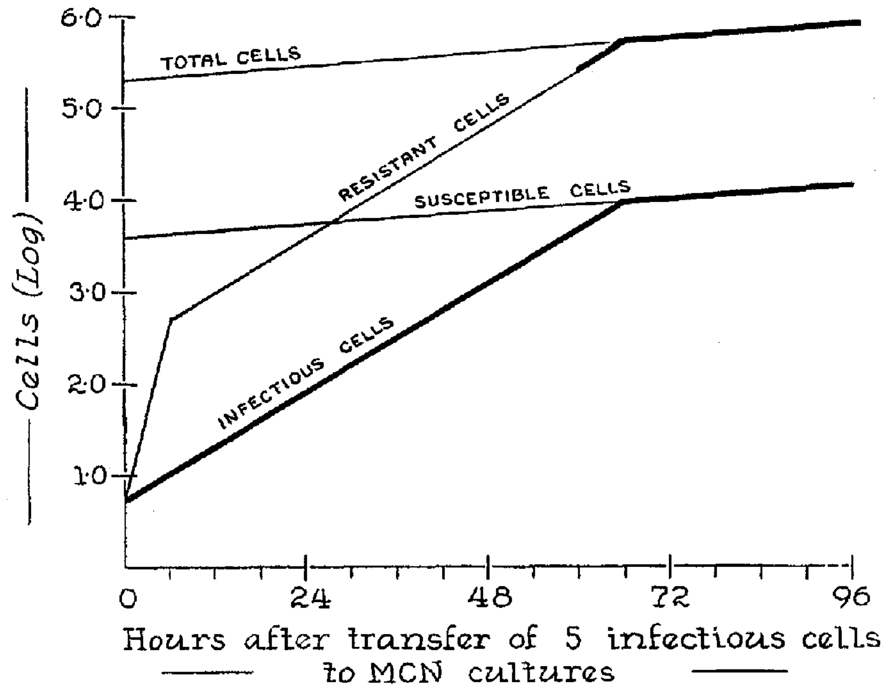

FIG. 4. Schematic presentation of the mode of spread of infection and of development of interference with VSV in MCN cultures following transfer of 5 virus-producing MCN_{NDV} cells.

the total number of cells increases from 2×10^5 to 8×10^5; the "receptive" cells from 4×10^3 to 1.6×10^4. The increase in NDV (virus-producing cells) is logarithmic, doubling every 6 hours until 66 hours when the line intercepts with the line of available receptive cells. Thereafter, the NDV titer rises slowly in accordance with the increase in cell population, and with it in new receptive cells. Disregarding the resistant cells transferred initially, the rise in cells rendered insusceptible to VSV is sharp in the first 6 hours but from then on parallels the increase in virus-producing cells at a 1.7 log higher level. This line intercepts the line of total cell population in 66 hours at which time the cultures are fully resistant to VSV. This schematic presentation does seem to fit, at least in principle, the data actually obtained in several experiments (see

Fig. 3 and Table V). The results will differ to some extent, of course, with variations in the number of "receptive" cells in given cultures as well as with variations in the total cell counts.

According to this scheme, variations in the susceptibility of cells at time of exposure and interference play dominant roles in maintenance of the persistent infection. No information is at hand at present as to the nature of the refractory state in the majority of the cells. This point, therefore, remains speculative. The role of interference, on the other hand, is well established and has been suspected also to participate in persistent infection in another cell-virus system (9). It remains to be shown, however, whether the virus-producing cells yield sufficient quantities of virus particles to account for development of interference within the established time limits. Preliminary experiments, in which aliquotes of 50 to 100 MCN_{NDV} cells were kept at 37°C. for periods of 4 to 12 hours before plating on monolayers of chick embryo fibroblasts failed to yield the expected number of plaques, although some increases in free virus were noted. It is probable on the other hand, that cells are not functioning optimally in dilute suspensions. This problem requires further study. Another possibility to account for the establishment of resistance in persistently infected cultures may be found in recent reports by Isaacs and Lindenmann (10, 11), who observed that interference is mediated in certain host-virus systems by a small viral component called interferon which escapes detection by conventional virological technics. Studies concerning this possibility are now in progress.

SUMMARY

Efforts were made to obtain information on some of the quantitative aspects of host cell-virus interactions in MCN cultures persistently infected with Newcastle disease, mumps, and 6-6 viruses, and to elicit the mechanism which permits simultaneous maintenance of virus and cells for indefinite periods of time.

It was shown by 4 different technics that only between 10 and less than 1 per cent of the cells yield infectious virus, depending upon the agent employed and, possibly, variations in the conditions of the cultures. No evidence was found to indicate production of non-infectious virus materials.

Only the cells carrying infectious virus are capable upon transfer to uninfected cultures to transmit the infection. Cells from persistently infected cultures, which are free of infectious virus at the time of transfer, failed to liberate virus at a later time during incubation periods of up to 4 weeks.

The virus-producing cells contain at any given moment not more than 1 infectious unit of virus, suggesting a linear mode of production; *i.e.*, as soon as a virus particle is completed, it is released.

Upon inoculation of MCN test tube cultures with chick embryo-adapted

NDV persistent infection and interference with vesicular stomatitis virus (VSV) is established with considerable delay. In contrast, following transfer of MCN-adapted NDV, in form of MCN_{NDV} cells or first allantoic passage seeds derived therefrom, the number of virus-producing cells increases logarithmically, doubling every 6 to 8 hours until a total of about 10^4 is reached. Thereafter their numbers rise in proportion to the increase in total cell population; *i.e.*, doubling approximately every 48 hours.

At the time when 10^4 virus-producing cells are present in the culture interference with VSV is solidly established. In order to obtain this result about 10^6 cells must have adsorbed virus particles, or, in other words, at least 10^6 virus units must have totally been produced instead of the 10^4 measured by infectivity assay.

The implications of these and previously reported data have been discussed in detail and a scheme of the course of events in persistently infected cultures has been presented.

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