STUDIES ON PERSISTENT INFECTIONS OF TISSUE CULTURES*

I. General Aspects of the System

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It has been observed repeatedly that cells in primary or continuous tissue culture may sustain inapparent as well as persistent infections with certain viruses (1–17). Thus, vaccinia, influenza, mumps, Newcastle disease, Eastern and Western equine encephalomyelitis, dengue, adeno-, poliomyelitis, Theiler's, and psittacosis viruses have been propagated in various tissue culture systems either without or with limited destruction of the host cells. In many of these studies changes in the cultural conditions were required in order to keep viral and cellular multiplication in balance. Antisera, viral inhibitors as present in normal sera, antiviral substances, or deficient media as well as low temperatures of incubation were required to maintain virus and cells in equilibrium. In some instances, persistent infection could be ascribed in part to the selection of an increasingly resistant host cell population.

In a study of 12 human cell strains in continuous tissue culture it was found that the lines fell into 2 groups (17, 18). Cells of group 1, of which the HeLa cells may be taken as the prototype, are epithelial in appearance and capable of supporting growth of a wide variety of viruses with the development of cytopathic effects. The MCN (19) and Lung-To cells (18), comprising group 2, are smaller (except for a few giants), round or irregularly shaped, and susceptible to only a limited number of viruses; *i.e.*, of the viruses tested only vaccinia, herpes simplex, and vesicular stomatitis viruses could be maintained in group 2 cells by serial passages with the appearance of lesions; the cells were incapable of supporting poliomyelitis, Coxsackie B, and adenoviruses; and, finally, mumps (Mps), Newcastle disease (NDV), and certain other members of the myxogroup propagated in the MCN or Lung-To cells, generally in the absence of recognizable cytopathic effects, and persistent infections were readily

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maintained without resort to manipulations of the cultural conditions. This last type of host cell-virus interaction has been studied extensively in order to discover the mechanism permitting both virus and cells to persist simultaneously in culture. The results will be presented in this and the accompanying reports.

Methods and Materials

Tissue Cultures.—Cell lines. The HeLa (20), MCN (19), and Lung-To (18) lines of human cells were employed. The strain of HeLa cells was originally obtained from the Microbiological Associates, Inc. The MCN line, kindly furnished by Dr. McCulloch, was derived from

TABLE I

Media Employed in Various Types of Tissue Cultures

Type of cell	Type of culture	Human serum	Horse serum	Basic medium Eagle diluted in:		Scherer's maintenance solution	Medium 199
				Hanks' balanced salt solution	Earle's balanced salt solution		
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
HeLa	Growth of stock cultures	20		80			
	Growth of tube cultures		10	90			
	Maintenance of tube and bottle cultures		10		90		
MCN	All cultures		10			60	30
Lung-To	All cultures		10			60	30

the bone marrow of a leukemic patient. The Lung-To line was developed in this laboratory from normal embryonic lung tissue obtained in form of a first passage plasma clot culture from Dr. Franklin of the Connaught Medical Research Laboratories (18). Stock cultures were maintained in 500 ml. Blake bottles and tube cultures were prepared according to the routine procedures established in this laboratory (17, 18).

Tissue Culture Media.—The various media employed are listed in Table I. The human sera were obtained from healthy blood donors through the Philadelphia Serum Exchange. The horse sera, as well as the various salt solutions, and synthetic media (in 10 or 100 × concentrated stock solutions) were purchased from the Cappel Laboratories, West Chester, Pennsylvania, and from the Microbiological Associates, Inc., Bethesda, Maryland. All sera and media were tested for toxicity in HeLa cell cultures prior to use. Penicillin and streptomycin (50 units and μg./ml., respectively) were added to all media. The pH was adjusted as routine to 7.4–7.6 by the addition of sodium bicarbonate, except in some experiments with vesicular stomatitis virus when a somewhat higher pH was desirable.

Persistently Infected Stock Cultures.—All infected MCN and Lung-To lines were maintained under exactly the same conditions as the uninfected parent strains. All cell lines were checked on each subculture for the presence or absence of virus.

Chick Fibroblast Monolayer Cultures.—These were prepared according to the procedure described by Dulbecco (21) and modified by Granoff (22): 10- to 12-day-old decapitated chick embryos were minced. The small tissue fragments were washed several times with Earle's balanced salt solution (EBS) and subjected to 3 to 4 30-minute periods of trypsinization on a magnetic stirrer (0.25 per cent trypsin in EBS). The resulting cell suspensions were poured through 2 stainless steel wire screens of decreasing mesh size, pooled, and kept at 4°C. until trypsinization was completed. The total cell suspension was then centrifuged at 1000 R.P.M. for 10 minutes and the supernatant was discarded. The sedimented cells were washed once with EBS, resuspended in complete nutrient medium (EBS containing lactalbumin hydrolysate—0.4 per cent, horse serum—20 per cent, 4.4 per cent solution of sodium bicarbonate—3.75 per cent) and filtered through gauze. The cells were counted in a hemocytometer and the suspension was adjusted to contain 8×10^6 cells/ml. Five ml. amounts of the suspension were seeded in 50 mm. Petri dishes and the plates were incubated at 37°C. in an atmosphere containing approximately 3 per cent CO_2. After 24 hours the cell sheets were washed once with EBS and inoculated with virus. The plates were then placed for 45 minutes at 37°C. to allow for virus adsorption and, subsequently, overlayed with 6.5 ml. of the agar-nutrient mixture kept at 44°C. The overlay consisted of EBS containing lactalbumin hydrolysate—0.5 per cent, yeast extract—0.1 per cent, gelatin—0.5 per cent, Difco's bacto-agar—1 per cent, sodium bicarbonate (4.4 per cent stock)—4.5 per cent, and neutral red at a concentration of 1:30,000. After solidification of the overlay the plates were inverted and incubated in a 3 per cent CO_2 atmosphere at 37°C. Plaques were counted on the 5th and 7th day following inoculation.

Clonal Growth of MCN Cells.—The procedures developed by Puck *et al.*, (23–25) were employed. Since in preliminary experiments it was found that the plating efficiency of MCN cells on glass was below 50 per cent, whereas, controls with Earle's L cells gave the expected 80 to 100 per cent values, all further experiments were performed on feeder layers of giant cells obtained by x-irradiation. Suspensions of HeLa or MCN cells at concentrations of 1.5×10^6 cells per ml. were irradiated in 2 ounce prescription bottles with 4000 r. A Machlett tube operating at 250 kvp (kilovolt peak) was used. With 0.5 mm. aluminum and 0.25 mm. copper filters the dose rate was calculated to be 122.5 per minute. The suspensions, therefore, were irradiated 2 times for 16 minutes each at an interval of 15 minutes. The cell suspension was redispersed by pipetting, diluted with complete medium to the desired concentration and 1×10^4 irradiated cells each were seeded into tubes. After 3 to 4 days when a loose network of "giants" had developed these cultures were used as feeder systems. The cells prepared for cloning by trypsinization with 0.01 per cent trypsin were suspended in complete medium and seeded on the feeder layers. Between 1 and 10 cells were added per tube in different experiments. The exact cloning efficiency of MCN cells with this method has not been established but it was found that feeder layers of HeLa cells gave better results than the homologous cells. The initial generation time of the cloned MCN and MCN_{NDV} cells was quite irregular. After 2 to 3 weeks of incubation clones of 20 to about 500 cells were observed, the clones of the persistently infected lines in general being smaller as compared to the parent MCN cells.

Viruses. Stock Preparations.—Pertinent information on the various viruses employed is presented in Table II. For stock virus production of egg-adapted strains 10- to 11-day-old chick embryos were inoculated by the allantoic route (NDV and influenza) with 0.2 ml. of virus diluted in nutrient broth, the inoculum containing 10^3 to 10^4 EID_{50}. Mumps-Po virus was grown in the amniotic cavity of 8-day-old chick embryos according to the technics described (27). The allantoic or amniotic fluids were harvested from chilled eggs after 48 hours or 6 days of incubation at 37°C., respectively. The fluids were pooled, distributed in 1 ml. amounts in ampuls, shell-frozen in a dry ice–alcohol bath and stored at −65°C.

Stocks of tissue culture strains of virus were prepared in 4- to 7-day-old Blake bottle cultures

of HeLa or MCN cells. These were refed before inoculation, and in addition, washed 3 times with Hanks's balanced salt solution if the growth medium had contained human serum. Medium with a pH of 7.6 to 7.9 was used for the production of VSV (vesicular stomatitis virus) in MCN cultures to achieve maximal virus yields. The virus inoculum (in Hanks's BSS) was generally 0.5 ml. containing approximately 10,000 TCD_{50}. The infected tissue culture fluids were harvested at 48 to 72 hours; *i.e.*, when cell destruction was nearly complete. The harvested material was centrifuged at 2000 R.P.M. for 20 minutes to sediment cell debris and the clear supernates were stored at −65°C. in sealed ampuls.

TABLE II

Stock Viruses

Virus	Strain	Host	Infectious units/ml.
Newcastle disease	Victoria	Chick embryo allantois	$10^{10.2}$ EID_{50}
		HeLa cells	$10^{7.2}$ TCD_{50}
Mumps	Po	Chick embryo amnion	$10^{5.2}$ TCD_{50}
		HeLa cells	$10^{3.7}$ TCD_{50}
6-6*	6-6	HeLa cells	$10^{6.2}$ TCD_{50}
Vesicular stomatitis	Indiana	HeLa cells	$10^{6.7}$ TCD_{50}
		MCN cells	$10^{8.2}$ TCD_{50}
Herpes simplex	96	HeLa cells	$10^{5.2}$ TCD_{50}
Influenza Type A	PR8	Chick embryo allantois	$10^{9.5}$ EID_{50}

* The origin of this virus is obscure. An agent was isolated in chick embryos from a patient with parotitis (case 6) and identified as mumps virus. After 6 amniotic passages it was transferred to HeLa cultures where it produced lytic lesions typical of mumps. In the 4th consecutive passage in HeLa cultures the titer suddenly increased more than 100-fold, yet the lesions remained the same. Back passage to chick embryos yielded no or low levels of hemagglutinins, although the amniotic fluids contained high titers of virus (10^{-8}) as determined by titration in HeLa cells. The agent was neutralized by over 90 per cent of human sera, many of which were free of antibodies to mumps. Guinea pig anti-mumps sera failed to neutralize it. The "6-6" agent has been identified in the meantime as a hemadsorption type 1 virus (26).

Titration of Viruses.—Titrations of viruses in form of infected allantoic, amniotic, or tissue culture fluids, infected intact MCN or Lung-To cells and infected cells frozen and thawed or sonically vibrated were performed in embryonated eggs, HeLa or MCN tube cultures, and on monolayers of chick embryo fibroblasts, depending on the conditions of the particular experiment. Suspensions of counted cells were frozen at −20°C. or in dry ice–alcohol and thawed at 37°C. several times in succession. Sonic disruption of cells was achieved by means of a 9,000 kc Raytheon magnetostriction oscillator using 15 to 30 ml. of cell suspension and periods of exposure up to 30 minutes. When intact cells were to be inoculated, the suspensions were constantly agitated by means of a magnetic stirrer while dilutions and inoculations were made. Dilutions of cells were made in 2- to 10-fold steps in complete medium, those of free virus in Hanks's solution. Using chick embryos, 3 to 10 eggs each were inoculated

with 0.2 ml. of one of the dilutions of virus or cell suspension by the allantoic (NDV) or the amniotic routes (mumps). The allantoic or amniotic fluids were harvested individually after 48 to 72 hours and 6 days, respectively, and tested for the presence of hemagglutinins. For titration in HeLa or MCN cells 2- to 4-day-old cultures were used which were refed prior to inoculation. In the case of VSV the pH of the MCN cultures was adjusted between 7.7 and 7.9 prior to inoculation. Between 2 and 10 tubes each were inoculated with 0.2 ml. of one of the dilutions. The inoculated cultures were observed for cytopathogenic effects at 1 to 2 day intervals and the lesions recorded. All infectivity titers were calculated by the method of Reed and Muench on the basis of hemagglutination or cytopathic effects. Monolayers of chick embryo fibroblasts were inoculated as a rule with 0.2 ml. of virus dilution or cell suspension unless stated otherwise. The inoculum was placed in the center of the plate and 2 to 8 cultures were used per dilution. The number of plaques on each plate was counted on the 5th and 7th day following inoculation.

Hemagglutination Tests.—The material to be tested was diluted with cold veronal saline in 2-fold steps using 0.4 ml. volumes. To each tube 0.2 ml. of a fresh 1 per cent suspension of chicken red cells (suspended in veronal saline) was added and the tubes were placed at 4°C. for 30 to 45 minutes, at which time the test was read. In cases in which only qualitative testing for presence of hemagglutinins was required (infectivity titrations) a few drops of the undiluted material was placed on a clean glass surface, 2 drops of 10 per cent chick red blood cells were added, and the mixture was agitated. If hemagglutinins were present clumping of cells took place in a matter of seconds.

Immune Sera.—Antisera to NDV, 6-6, and VSV were obtained in guinea pigs weighing between 600 and 800 gm. All animals were bled prior to immunization. The NDV inocula consisted of infected allantoic fluids ($10^{10.2}$ EID_{50}/ml.). The 6-6 and VSV inocula were derived from monkey renal cells which were grown on a mixture consisting of 98 per cent medium 199 and 2 per cent calf serum. Before inoculation the nutrient fluid was changed to 100 per cent medium 199. These preparations contained $10^{7.8}$ and $10^{6.3}$ TCD_{50}/ml., respectively. Three intraperitoneal doses of 1, 2, and 3 ml. of NDV and 6-6 were given at 1 to 2 week intervals. The animals were bled 10 days after the last dose. In the case of VSV only 2 doses were given, the first containing 10^2, the second $10^{6.3}$ TCD_{50}. These animals possessed neutralizing titers greater than 1:256 at the time of the second dose and were bled out, therefore, 10 days thereafter. The immune sera were tested for neutralizing antibodies using essentially the technic described previously for mumps virus (28). The anti-NDV and 6-6 sera were assayed in HeLa cultures with HeLa cell–adapted viruses, the anti-VSV sera in MCN cells using an MCN-adapted strain of the agent. None of the pre-immunization sera revealed neutralizing activity. Following immunization the anti-NDV serum pool neutralized 1000 TCD_{50} in dilution 1:512; the anti-6-6 pool—100 TCD_{50} in dilution 1:1024; and the anti-VSV serum—1000 TCD_{50} in dilution 1:4096. For experiments with mumps virus a human hyperimmune serum pool was used which was obtained from the Philadelphia Serum Exchange. This serum neutralized 1000 TCD_{50} in dilution 1:512.

EXPERIMENTAL

Persistent Infections of MCN and Lung-To Cells

Inoculation of chick embryo– or HeLa cell–adapted mumps, Newcastle disease (NDV), or 6-6 viruses into MCN or Lung-To cultures may or may not produce some slight lesions, depending upon the concentration of the infectious agent. If the inoculum contained more than 10^6 EID_{50} or TCD_{50}, as determined by titration of the seed in chick embryos or HeLa cells, some cellular destruc-

tion was noted in many instances but the cultures rapidly recovered. Since test tube cultures of MCN or Lung-To cells at the time of inoculation, as a rule, do not contain more than 2 to 4 $\times$ 10^5 cells, it would seem that cytopathic effects are produced only when the number of virus particles added exceeds the number of cells. Even though some lesions may be observed following the initial inoculation of virus under the described conditions, second and further passages, performed by transfer of medium or medium and cells from the infected

TABLE III

Serial Passages of NDV and 6-6 Viruses in MCN Cultures

Virus	Passage No.	Virus titer		Days of incubation per passage	Dilution used for next passage	Total days of incubation	Total dilution of original inoculum
		Direct (CPE)	Sub-inoculation of HeLa cultures TCD_{50}				
NDV* (Victoria)	1	$10^{3.2}$‡	$10^{6.2}$	3	10^{-1}	3	$10^{-0.7}$
	2	$<10^{0.7}$	$10^{5.2}$	5	10^{-3}	8	$10^{-2.4}$
	3	$<10^{0.7}$	$10^{2.2}$	8	10^{0}	16	$10^{-5.1}$
	4	$<10^{0.7}$	$10^{3.2}$	6	10^{-2}	22	$10^{-6.8}$
	5	$<10^{0.7}$	$10^{4.2}$	4	10^{-2}	26	$10^{-9.5}$
	6	$<10^{0.7}$	$10^{4.2}$	7	10^{-1}	33	$10^{-12.2}$
	7	$<10^{0.7}$	$10^{2.7}$	6		39	$10^{-13.9}$
6-6§	1	$10^{1.2}$‡	$10^{6.2}$	3	10^{-1}	3	$10^{-0.7}$
	2	$<10^{0.7}$	$10^{3.2}$	4	10^{0}	7	$10^{-2.4}$
	3	$<10^{0.7}$	$10^{3.2}$	4	10^{0}	11	$10^{-3.1}$
	4	$<10^{0.7}$	$10^{3.7}$	4	10^{-2}	15	$10^{-3.8}$
	5	$<10^{0.7}$	$10^{3.2}$	6	10^{-1}	21	$10^{-6.5}$
	6	$<10^{0.7}$	$10^{4.2}$	8	10^{-2}	29	$10^{-8.2}$
	7	$<10^{0.7}$	$10^{3.2}$	13		42	$10^{-10.9}$

* 19th passage in HeLa cells.

‡ Transitory, partial lesions, cultures recovered on prolonged incubation.

§ 14th passage in HeLa cells.

cultures to fresh MCN or Lung-To tubes, regularly failed to produce cytopathic effects. Yet, the continued presence of virus could readily be detected by titration of the passage materials in chick embryos or HeLa cells. Table III presents examples of such passage series. It is evident that the viruses actually multiplied by considering the dilution factors incident to each passage and by comparing the titers of the stock viruses initially inoculated with those obtained in consecutive transfers. For example, in the NDV series $10^{7.2}$ infectious doses for HeLa cells were administered in the first passage; yet, after 7 serial transfers, representing a dilution factor of the initial inoculum of $10^{-13.9}$, titration of the last passage material in HeLa cells yielded a virus concentration of $10^{2.7}$ infectious doses per ml.

The results of these passage series indicated that both viruses and cells could be maintained simultaneously in cultures. Indeed, MCN tubes of the 4th passage of NDV or the 6-6 virus were kept and maintained by frequent feeding until the cultures became crowded. At this stage the cells were removed from the glass surface by trypsinization and reseeded in small bottles where they grew out to form sheets. Titration of the media in HeLa cells revealed the presence of virus. From then on the infected cultures could be maintained in

TABLE IV

Persistence of Viruses in MCN and Lung-To Cultures

Cell line	Virus originally introduced								
	NDV (Victoria)			Mumps (Po)			6-6		
	Cell passage No.*	Total days after introduction of virus	TCD_{50} of virus per ml.‡	Cell passage No.	Total days after introduction of virus	TCD_{50} of virus per ml.	Cell passage No.	Total days after introduction of virus	TCD_{50} of virus per ml.
MCN	2	29	$10^{1.2}$	2	23	$10^{2.2}$	2	26	$10^{3.2}$
	8	104	$10^{2.2}$	4	76	$10^{1.7}$	9	73	$10^{3.2}$
	10	179	$10^{2.7}$	8	135	$10^{2.2}$	13	199	$10^{4.2}$
	14	262	$10^{3.2}$	12	207	$10^{2.2}$	15	240	$10^{5.2}$
	18	316	$10^{4.7}$				18	320	$10^{5.2}$
	20	358	$10^{2.7}$				21	362	$10^{2.7}$
	25	435	$10^{2.7}$				26	439	$10^{3.7}$
Lung-To	2	49	$10^{1.2}$	2	23	$10^{2.2}$	2	49	$10^{2.7}$
	4	72	$10^{2.2}$	4	76	$10^{1.7}$	4	77	$10^{2.2}$
	6	179	$10^{4.2}$	8	135	$10^{2.2}$	6	179	$10^{3.2}$
	9	235	$10^{3.2}$	12	207	$10^{3.2}$	9	235	$10^{2.7}$
	18	468	$10^{4.7}$				18	468	$10^{5.2}$

* Cell passages were made at 2 to 3 week intervals; between passages the media were renewed every 4 to 7 days.

‡ Media or media and cells frozen and thawed once were assayed in HeLa cells.

Blake bottles according to the same growth and maintenance schedules as employed for the uninfected parent cells. These two sublines, referred to as MCN_{NDV} and $MCN_{6\text{-}6}$ have been maintained now for over 18 months. The pertinent data are recorded in Table IV. It is apparent that virus was found at all times tested in concentrations ranging from $10^{1.2}$ to $10^{5.2}$ TCD_{50}/ml., as assayed in HeLa cells. These amounts are low when compared to the number of cells available in the bottle cultures, which varied from 10^7 to $10^{7.5}$. Since Blake bottle cultures contain 25 ml. of medium, the total quantity of virus maximally detectable amounted to $10^{6.6}$ TCD_{50}. Thus, considerably less than one virus unit was found per cell at any given time of assay. In subsequent experiments persistent infection was established directly in bottle cultures of

MCN or Lung-To cells by addition of mumps (Po), Newcastle disease, or 6-6 viruses. The results of some of these tests are also included in Table IV.

The virus titers found in persistently infected cultures varied over a more than 100-fold range, suggesting fluctuations in the degree of susceptibility of the cultures from time to time. It was noted in addition that the titers differed when either the cell-free media, removed at the times of refeeding, or the cell suspensions, used for reseeding were tested. For this reason, the media and cells were assayed separately in further experiments. After decanting of the

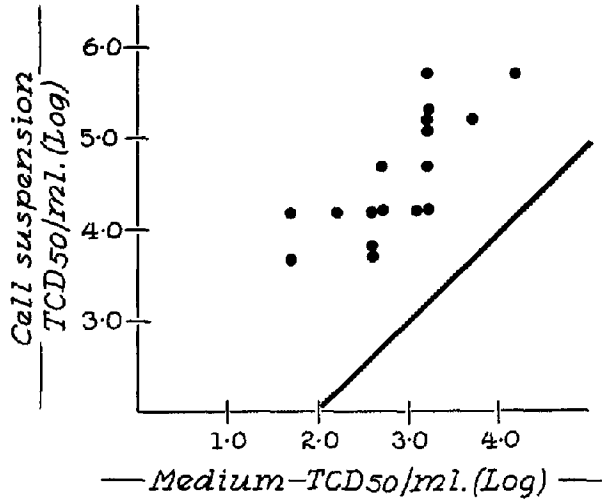

FIG. 1. Relation between virus titers in media and cell suspensions derived from MCN_{NDV} cultures.

culture fluids they were centrifuged at 2000 R.P.M. for 20 minutes to sediment floating cells and the supernates were saved. The cells were then removed from the glass by trypsinization, washed 3 times in Hanks's solution, and finally suspended in fresh medium to the original volume. The results of 15 comparative titrations of medium and cells of MCN_{NDV} cultures are presented graphically in Fig. 1. It can be seen that the medium contained at most 10 per cent of the amount of virus found in the cells and frequently not more than 1 per cent. Corresponding studies with the other persistently infected cell lines gave similar results.

Attempts to detect hemagglutinating or viral complement-fixing activity in media or disintegrated cell suspensions failed to meet with success. The amounts of virus determined by infectivity titrations were obviously too low to be detectable by these technics. These results indicate that incomplete virus reproduction, as observed with certain strains of influenza virus (17, 29), did not occur to any significant extent with the viruses employed here.

Attempts to Activate the Infectious Process by Various Manipulations.—It

was thought that the presence of 10 per cent horse serum in the media possibly exerted an inhibitory effect upon the various viruses and thereby permitted the survival of the cultures. A reduction in the concentration of horse serum to 2 or 1 per cent, or even complete omission did not alter the results. The carrier lines initially remained healthy but then degenerated after 7 or more days, at a time when non-infected cultures, maintained under the same deficient conditions, also were lost.

In an effort to determine the effect of ultraviolet irradiation upon persistently infected and uninfected cultures, monolayers of cells were exposed in open Petri dishes to a General Electric germicidal lamp at a distance of 7 inches for periods ranging from 1 second to more than 3 minutes. Infected and non-infected cultures did not reveal any differences in susceptibility and the degrees of destruction increased with the prolongation of exposure to ultraviolet light to the same extent in both. There were no increases in virus titers following irradiation of infected cultures. On the contrary, part of the virus was inactivated on prolonged exposure to ultraviolet. Thus, no evidence was obtained to suggest induction as observed in certain lysogenic bacterial cultures.

Changes in the Properties of Persistently Infected Cultures

Growth Rates of Cells.—It has been pointed out above that the persistently infected culture lines could be maintained on the same routine as the non-infected parent cultures with respect to feeding and reseeding. These routine procedures are designed for convenience of handling at appointed days rather than for optimal growth rate and yield of cells. By observation of the development of cell sheets in bottle cultures the impression was gained that the infected lines grew at a somewhat slower rate. It was essential, therefore, to establish the growth rates of cells from normal and persistently infected cultures in order to determine whether or not the presence of virus exerted an adverse effect. Fig. 2 presents an example of such experiments.

Cells from Blake bottle cultures of the MCN, MCN_{NDV}, and MCN_{6-6} lines were suspended by adding trypsin to the medium and incubation for 10 minutes at 37°C. The suspensions were centrifuged at 800 R.P.M. for 15 minutes and the cells resuspended in 25 ml. of fresh medium. After counting of the cells the suspensions were adjusted to contain 1×10^5 cells/ml. and more than 40 tubes each were seeded with 1 ml. of the preparations. Four tubes of each group were removed from the incubator at 24 hours and at varying intervals thereafter. In order to maintain the cultures in good condition 0.5 ml. of fresh medium was added to the remaining tubes on the 7th and 15th day. The cells were suspended by the addition of 1 ml. of 0.5 per cent trypsin and the contents of the 4 corresponding tubes were pooled. Trypan blue was added to differentiate between viable and non-viable cells. The latter amounted always to less than 5 per cent. The cell counts obtained were adjusted to allow for the dilution factors introduced by the addition of medium and trypsin.

It is seen in Fig. 2, that the control MCN cells multiplied at a logarithmic rate for 13 days, with a generation time of about 48 hours. Thereafter, the curve fell off sharply and it is evident that 3×10^6 cells represent the maximum

yield that can be obtained in the culture tubes under the conditions employed. The cells of persistently infected cultures initially propagated at similar rates, the MCN_{NDV} cells for 5, and the MCN_{6-6} cells for about 9 days. The rates then decreased significantly and the ultimate yields were 50 and 30 per cent less than those of the uninfected series. The MCN_{NDV} cells revealed infectivity titers of $10^{3.0}$ and $10^{2.5}$ TCD_{50} at the start and the end of the experiment, respectively. The corresponding figures for the MCN_{6-6} cells were $10^{4.0}$ and $10^{5.0}$.

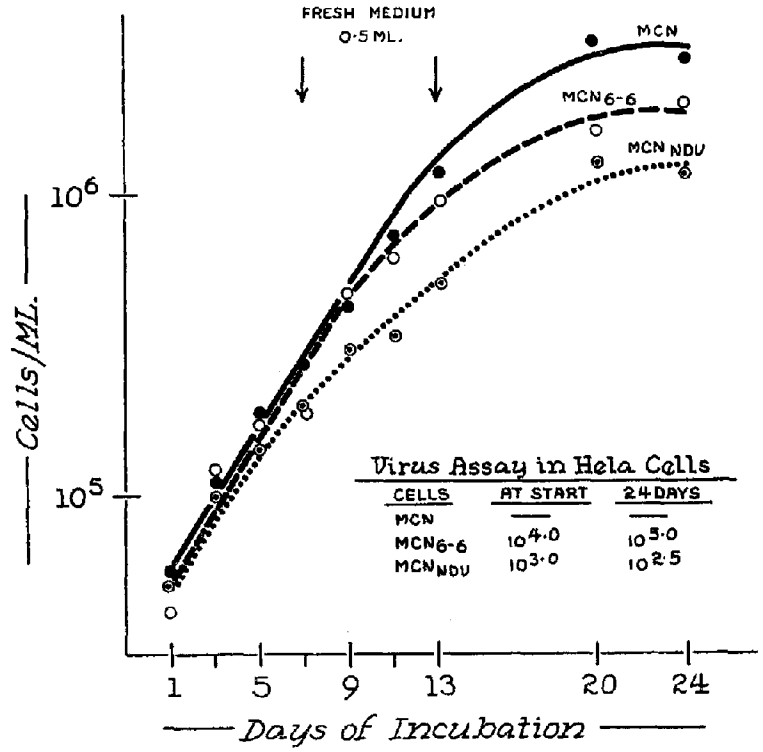

FIG. 2. Comparative growth rates of MCN, MCN_{NDV}, and MCN_{6-6} cells

Efforts were made to obtain growth rates of normal and persistently infected MCN cells in suspended cultures using the technic of Graham and Siminovitch (30). Although the various cells could be propagated in this fashion, only the uninfected MCN cultures gave reliable cell counts with generation times of about 48 hours. Cells from persistently infected cultures, on the other hand, tended to aggregate so that the counts obtained varied considerably. No method has been found as yet to avoid this aggregation.

Changes in Metabolism in Persistently Infected Cultures.—Studies on respiration and anaerobic glycolysis failed to reveal differences between MCN,

MCN_{NDV}, and MCN_{6-6} cells. On the other hand, aerobic glycolysis was significantly affected by the presence of virus, and 3 to 4 times the amount of lactic acid was produced in MCN_{NDV} cultures as in the parent MCN cells. The MCN_{6-6} cells also showed a significant though less marked increase in aerobic glycolysis. These experiments have been recorded in detail elsewhere (31).

TABLE V

Comparative Titrations of VSV in Normal and Persistently Infected MCN and Lung-To Cultures

Cells	Dilution of VSV	Persistent infection																			
		None					NDV					Mumps, Po					6-6				
		Day after challenge																			
		1	2	3	5	7	1	2	3	5	7	1	2	3	5	7	1	2	3	5	7
MCN	10^{-1}	4*	4	4	4	4	3	4	4	4	4	3	4	4	4	4	2	4	4	4	4
	10^{-2}	3	4	4	4	4	±	2	3	3	3	0	±	1	2	2	0	1	1	3	3
	10^{-3}	1	4	4	4	4	0	0	±	1	1	0	0	0	0	1	0	0	0	0	0
	10^{-4}	1	3	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10^{-5}	1	2	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10^{-6}	0	1	3	4	4															
	10^{-7}	0	1	2	4	4															
	10^{-8}	0	0	0	0	0															
Lung-To	10^{-1}	4	4	4	4	4	3	3	3	3	3	1	2	2	3	4	2	3	4	4	4
	10^{-2}	3	4	4	4	4	±	±	1	2	3	0	1	1	2	3	0	2	2	3	4
	10^{-3}	1	3	4	4	4	0	0	0	±	1	0	0	0	0	0	0	0	1	2	3
	10^{-4}	1	1	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10^{-5}	0	±	3	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10^{-6}	0	0	1	4	4															
	10^{-7}	0	0	0	0	0															
	10^{-8}	0	0	0	0	0															

* 4, complete destruction of cells; 0, no lesions; ± to 3, intermediary stages of cytopathic effects.

Changes in Susceptibility to Cytopathogenic Viruses.—It was pointed out above that the MCN or Lung-To cells are susceptible to vaccinia, herpes simplex, and vesicular stomatitis (VSV) viruses, and that these cells are destroyed as a result of such infections. As a matter of fact, the cells were particularly suitable for VSV in that the amount of virus produced exceeded that obtained in other cell lines, such as the HeLa cells, by a factor of 10 to 100 (17). It was of considerable interest, therefore, when it was noted that the persistently infected cultures revealed a high degree of resistance to VSV. Table V presents representative examples of comparative titrations of VSV in MCN, MCN_{NDV}, MCN_{Mps}, and MCN_{6-6} cells, as well as in the corresponding Lung-To

cultures. A summary of numerous tests is given in Table VI. It is seen that the VSV titers were reduced by as much as 7 $\log_{10}$ units. Furthermore, it is noteworthy that the VSV infection always leads to complete destruction (4+) in MCN or Lung-To cells, whereas, in persistently infected cells only partial

TABLE VI

Summary of Titrations of VSV in Persistently Infected Cultures

Cells	No. of tests	Results of VSV titrations (4 day reading)	
		TCD_{50}/ml.* range	Protective index range
MCN	12	$10^{7.2}$–$10^{9.2}$	0
MCN_{NDV}	12	$10^{1.2}$–$10^{4.2}$	$10^{3.5}$–$10^{7.0}$
MCN_{Mps}	4	$10^{1.2}$–$10^{3.7}$	$10^{4.0}$–$10^{6.0}$
MCN_{6-6}	4	$10^{1.2}$–$10^{4.2}$	$10^{3.5}$–$10^{5.0}$
Lung-To	4	$10^{7.2}$–$10^{8.2}$	0
Lung-To_{NDV}	2	$10^{2.2}$–$10^{3.2}$	$10^{3.5}$–$10^{4.5}$
Lung-To_{Mps}	3	$10^{1.2}$–$10^{4.2}$	$10^{3.5}$–$10^{6.0}$
Lung-To_{6-6}	2	$10^{3.2}$–$10^{3.7}$	$10^{4.0}$–$10^{4.0}$

* The destruction of MCN or Lung-To cells by VSV always goes to completion (4+). In persistently infected cells often only partial degeneration is obtained. In these cases the end points were based upon 2+ or greater destruction of the cultures.

TABLE VII

The Production of Non-Infectious Hemagglutinins in MCN, MCN_{NDV} and MCN_{6-6} Cultures Following Inoculation of Influenza A (PR8) Virus

Cells	Inoculum			
	PR8		Normal allantoic fluid	
	Hemagglutinin titers			
	Hrs. after inoculation			
	2	22	2	22
MCN	<1:2	1:96	<1:2	<1:2
MCN_{NDV}	<1:2	1:2*	<1:2	<1:2
MCN_{6-6}	<1:2	1:2*	<1:2	<1:2

* Atypical pattern

degeneration may be noted at the endpoint dilutions. In these cases a 50 per cent destruction (2+) was taken to calculate the titration endpoints. As will be shown in the succeeding paper of this series (32), VSV is highly sensitive to a lowering of the pH in the culture medium. It was essential, therefore, to renew the medium of the cultures to be challenged just prior to inoculation and to adjust the pH to 7.7–7.9. This was done in all the experiments presented in

Tables V and VI. All persistently infected cultures, be they MCN or Lung-To carrying NDV, 6-6, or mumps viruses, resisted VSV infections to significant though variable extents.

The carrier cultures resisted infection not only by VSV but to some extent also by other viruses; *i.e.*, herpes simplex and influenza A (PR8) viruses. With respect to the latter, it has been reported that MCN and Lung-To cells support the production of non-infectious hemagglutinins (NIHA), but not of fully infectious progeny (17).

Bottles of mature MCN, MCN_{NDV}, and $MCN_{6\text{-}6}$ cultures were inoculated with 10^9 EID_{50} of PR8 virus or normal allantoic fluid as control. After incubation for 2 hours the inocula were removed and the cultures were washed 3 times with Hanks's solution. One bottle of each series was harvested at this time, the others were incubated for an additional 22 hours. The cells were scraped into the media and then disintegrated by sonic vibration.

The results of the hemagglutination test are shown in Table VII. The infected MCN culture yielded an NIHA titer of 1:96, whereas in the MCN_{NDV} and $MCN_{6\text{-}6}$ preparations agglutination was noted only in the 1:2 dilutions, and the red cell patterns observed were atypical, suggesting that cellular debris or other non-specific factors prevented the proper settling of the erythrocytes.

Attempts to Cure Persistently Infected Cultures by Specific Immune Sera

Several experiments were carried out in efforts to cure persistently infected cultures by addition of specific immune sera to the media. In the first two to be described the fate of the carried viruses was evaluated by infectivity titrations in HeLa cells.

Bottle cultures of MCN_{NDV} and $MCN_{6\text{-}6}$ cells were prepared and to the medium of one set homologous guinea pig immune serum was added, to that of the other set normal guinea pig serum. The anti-NDV serum neutralized 1000 TCD_{50} of virus when diluted 1:512. The final concentration of this serum in the medium was 1:20. The anti-6-6 serum, which neutralized 100 TCD_{50} of the homologous virus in dilution 1:1024, was added to the cultures in a final concentration of 1:30. The 2 sets of cultures in the 2 respective experiments were then maintained in the usual manner, by refeeding and reseeding of the cells at appropriate intervals, using each time the corresponding sera as an addendum to the media. At varying intervals, usually at the time of reseeding, aliquots of media and cells of the 2 series were titrated in HeLa cells for the presence of virus. Sublines were made at intervals in small bottles or test tubes. After these were established the immune or normal sera were removed from the media of part of the cultures in order to determine the reappearance of virus in time by repeated titrations for NDV or 6-6 virus respectively.

It can be seen from Fig. 3 that the immune sera prevented the detection of any virus in the media throughout the periods of treatment of the cultures, which ranged from 95 to 137 days. The cell suspensions, likewise, failed to yield evidence of the presence of virus, except for small amounts during the first 3 or 4 weeks following onset of treatment. The normal sera had no effects

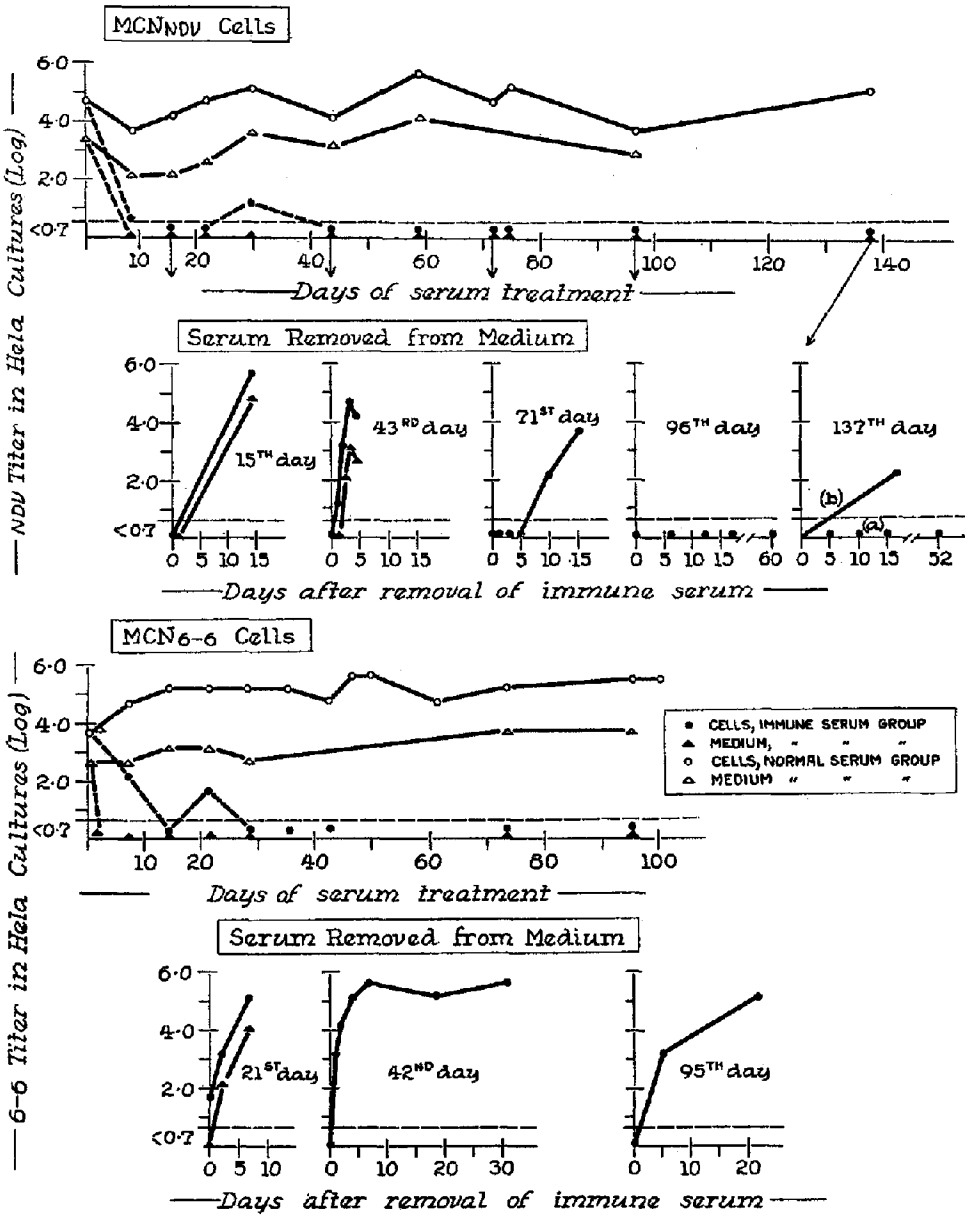

FIG. 3. The effect of specific immune sera on persistent infection of MCN cultures with Newcastle disease and 6-6 viruses as determined by titration in HeLa cultures.

and the NDV or 6-6 titers ranged between $10^{2.2}$ and $10^{4.2}$ in the media, and from $10^{3.7}$ to $10^{5.2}$ in the cell suspensions. When the immune sera were removed from the cultures at the indicated intervals the viruses reappeared in most instances and regained gradually titers corresponding to those seen in the normal serum

TABLE VIII

Effect of Mumps Hyperimmune Serum on Persistence of Virus in MCN_{Mps} Cultures as Assayed by Resistance to VSV

Total period of serum treatment		Days after removal of serum	Resistance to VSV*	
Days	Concentration of sera		Anti-serum group	Normal serum group
9	1:20	—	+*	+++
10	1:20	0	++	+++
		6	++++	++++
21	1:20	0	++	++++
		10	++++	++++
130	1:20	2	++++	++++
142	1:20	—	+	++++
143	1:20	0	++	++++
		1	+++	++++
		2	++++	++++
159	1:5	1	+++	++++
		2	++++	++++
168	1:5	0	++	++++
		3	++++	++++
172	1:5	0	+	++++
		2	++	++++
		7	++++	++++
187	1:5	5	++++	++++

*+, reduction in VSV titer by 1 $\log_{10}$ units.
++, " " " " " 2 " " .
+++, " " " " " 3 " " .
++++, " " " " " 4 or more $\log_{10}$ units.

controls. It was apparent, however, that the re-establishment of the virus required more time as the period of cultivation in the presence of immune serum was extended. Indeed, one of the immune serum lines of the MCN_{NDV} series was finally cured after 96 days of treatment. When the antibodies were removed at this time, virus failed to reappear within 60 days thereafter. A

retest of this subline after 137 days again did not yield NDV after the removal of antiserum. However, another subline of MCN_{NDV} cells carried in the presence of anti-NDV for 137 days, revealed virus 17 days after omission of the antiserum from the media. The MCN_{6-6} cultures were not cured in 95 days of treatment. It is of interest to note that the one cured MCN_{NDV} culture was fully susceptible to VSV and persistent infection could readily be re-established upon inoculation with NDV.

In an additional experiment, attempts were made to determine the effect of immune serum by loss in resistance to VSV. MCN_{Mps} cultures were maintained, as described for the above experiments, in the presence of human mumps hyperimmune serum or human control serum free of mumps antibodies. The immune serum neutralized 100 TCD_{50} of the Po strain in dilution 1:512. It was added to the medium initially in a final concentration of 1:20, and later in 1:5. Assays for mumps virus in the cell suspensions of the normal serum group yielded titers of $10^{1.7}$ to $10^{3.2}$ TCD_{50}. The media of the immune serum groups were throughout negative but the cells retained detectable though low titers up to the 144th day. At this point, the amount of immune serum was increased to a final concentration of 1:5. Thereafter, virus was no longer directly detectable in the cells. Test tube cultures from the 2 groups were prepared at intervals ranging from the 9th to the 187th day of serum treatment. These were grown initially in the presence of immune sera until the cell sheets had formed but then the antibodies were removed. These cultures were challenged with 100 to 1000 TCD_{50} of VSV either when the immune serum was still present or at various days after its removal. It is evident from Table VIII that the resistance was greatly reduced when the anti-mumps serum was present. This serum had no neutralizing effect on VSV. When the cultures were challenged immediately after removal of the serum their resistance, as a rule, also was low. However, in the ensuing days resistance was readily re-established.

These data, as well as corresponding experiments with persistently infected Lung-To cells, showed that immune sera had some effect but rarely cured the cultures completely.

Analysis of the MCN Cell Populations

The above section showed that persistent infection of MCN or Lung-To cultures led to a decrease in the growth rates of the cells, an increase in aerobic glycolysis and the development of resistance to cytopathogenic viruses. It was conceivable that these changes reflect a selection of cells, either immediately after inoculation of virus or in the course of persistent infection. Indeed, as pointed out above, addition of Newcastle disease, 6-6, or mumps viruses into MCN or Lung-To cultures at multiplicities of greater than 1, causes some cellular destruction. With smaller doses no evidence was found of cytopathic

effects or losses in cell counts. However, in these cases the number of cells destroyed was possibly too small to be recognized. It was thought that the cloning technic described in the section on methods might provide pertinent information. If a proportion of the cell populations were fully susceptible, some of the clones derived therefrom should be completely destroyed by NDV or the other viruses. Other cloned lines should possibly be resistant to these agents and incapable of supporting persistent infections. A total of 50 cloned MCN lines were obtained. None of these showed any cytological differences, nor did anyone of them degenerate following the addition of NDV. All became persistently infected as shown by subinoculation of HeLa cells at varying intervals or by establishment of resistance to VSV. Prior to the infection with NDV, all cloned lines were fully susceptible also to VSV. Thus, it would seem to be evident that the populations of MCN cultures are not composed of cells inherently susceptible or resistant to NDV (Table IX). It is suggestive rather that a cell has to

TABLE IX

Summary of Results Obtained with Cloned Lines of MCN and MCN_{NDV} Cells

Clones		Persistently infected	Susceptibility to VSV	Establishment of persistent NDV infection
Cell type	No. of clones			
MCN	50	0	50	50
MCN_{NDV}	39	0	39	39

be in a certain physiological or nutritional state to be capable of yielding infectious virus. Those not in this state at the time of infection are merely rendered resistant to VSV without yielding other direct evidence of infection, such as the production of non-infectious hemagglutinins or complement-fixing antigens.

Similar experiments were carried out with MCN_{NDV} cells except that anti-NDV serum was added to the cell suspension to neutralize extracellular virus. Cloning was readily achieved, although the initial growth was somewhat slower than that observed with MCN cells. This was not unexpected in view of the decrease in growth rates. The 39 lines of MCN_{NDV} cells so obtained were morphologically alike and did not differ from the MCN lines derived from uninfected cells. As shown in Table IX, none of these were shown to contain NDV. All were fully susceptible to VSV and upon inoculation with NDV developed persistent infections. As mentioned above, cultures cured of persistent infection by antiserum lost their resistance to VSV and could also be reinfected with NDV. These data indicate that persistent infection does not lead to a selection of cells. No differences in behavior was found among cloned cell lines derived from MCN and MCN_{NDV} cultures.

Changes in the NDV Strain on Persistence in MCN Cultures

The persistent infections in MCN or Lung-To cells were originally established with seed viruses derived either from infected HeLa cultures or chick embryos. It became apparent that the NDV strain underwent some changes as a result of its maintenance in MCN cultures. The other myxoviruses have not been studied as yet with this respect.

It was noted that in monolayers of chick embryo fibroblasts MCN_{NDV} cells produced plaques which were small and fuzzy in contrast to the large and clear ones of the chick embryo—adapted parent virus. The plaque size remained small when the virus from MCN_{NDV} cultures was passaged once through chick

TABLE X

Comparison of Various Preparations of the Victoria Strain of NDV

NDV Preparation	Titration in				
	Chick embryos	HeLa cultures	Monolayers of chick embryo fibroblasts	MCN cultures	
				1st Passage	2nd Passage
	EID_{50}/ml. log	*TCD_{50}/ml. log*	*No. of plaques/ml. log*	*TCD_{50}/ml.* log*	*TCD_{50}/ml. log*
Chick embryo–adapted	9.3	8.2	8.4	2.2	<0.7‡
HeLa cell–adapted	6.3	6.2	6.15	1.2	<0.7
MCN_{NDV} cells, sonically disrupted	4.7	4.2	4.35	1.7	<0.7
1st allantoic passage of MCN_{NDV}	9.2	7.7	8.6	3.2	<0.7

* Based on 2+ cytopathic effect.

‡ No lesions observed in any of the 2nd passages.

embryos by the allantoic route. The original egg-adapted virus produced mainly plaques with a diameter of about 2 to 3 mm., and only a rare plaque was smaller in size. The MCN_{NDV} inocula produced only pin-point plaques and none of the larger size was found. The HeLa-adapted virus gave plaques of intermediate size.

In view of this observation, it was of importance to see whether the NDV strains after passage through MCN cultures differed from the parent egg-adapted virus with respect to their cytopathogenicity for MCN cells. The results of comparative titrations of 4 NDV preparations are presented in Table X. The Victoria strain maintained in chick embryos or in HeLa cultures as well as a suspension of sonically disintegrated MCN_{NDV} cells and a first allantoic fluid passage of virus from the persistently infected cultures were titrated in chick embryos, HeLa cells, monolayers of chick embryo fibroblasts, and MCN cells. The results of the first 3 methods gave, with few exceptions, comparable

results. The allantoic fluid seeds gave possibly somewhat higher titers in chick embryos than in the other hosts. In MCN cells significant, though incomplete cytopathic effects were seen in all 4 series (not more than 2+) with the more concentrated inocula. It was of interest that the sonically disrupted MCN_{NDV} preparation, in spite of a low infectivity titer, produced some cellular destruction. However, on 2nd passage in MCN cells cytopathogenicity was no longer apparent.

These results did not indicate that the NDV strains passed through MCN cells behave differently from the chick embryo or HeLa-adapted virus with respect to cytopathogenicity. On the other hand, considerable differences were noted with regard to the establishment of persistent infection in MCN cells which will be recorded in the third paper of this series (33).

DISCUSSION

The data presented show that persistent, inapparent infections with Newcastle disease, 6-6, and mumps viruses can readily and reproducibly be established in MCN and Lung-To cultures. Such cultures have been maintained by refeeding and reseeding of the cells at intervals for more than 18 months on the same schedules as employed for the uninfected parent lines. Virus was detectable at all times tested by subinoculation of susceptible hosts. No changes in cultural procedures were required to achieve these results, whereas in many other host cell-virus systems persistent, inapparent infections resulted only in the presence of specific viral antibodies, or inhibitors as present in normal sera, or when deficient media or low temperatures of incubation were employed (3, 6, 7, 13–15). In the present studies the media contained horse serum which could have provided inhibitors for the myxoviruses involved. However, reduction in or removal of the serum did not convert the inapparent into overt infections.

The viruses in the persistently infected cultures were associated mainly with the cells and only 10 per cent or less of the agents was found free in the media. The total virus concentration was always relatively low in comparison to the number of cells present in the cultures, suggesting that only a fraction of the cells were producing virus. This was found to be true in other experiments to be reported in the third paper of this series (33). No hemagglutinating or viral complement-fixing activities were detectable in the infected cultures in keeping with the low titers of infectious virus observed. The absence of these activities may be taken to denote that little, if any, incomplete virus was formed.

While the infectivity data suggested that only few cells were involved in the persistent infections, other observations showed that nearly all cells were affected in some way by the presence of the viruses. This is evident from the fact that the carrier cultures as compared to the uninfected parent cells revealed lower growth rates and final yields of cells, an increased aerobic glycolysis (31), and marked resistance to certain cytopathogenic viruses. It will be shown in

the second paper of this series (32) that the resistance observed represents an example of viral interference.

Attempts to cure cultures of the persistent infection by antiserum met only with partial success. While the virus titers decreased to considerable extents, the removal of sera after continuous treatment of cultures for up to 187 days led, in most instances, to the reappearance of virus. Only one culture was finally cured. It is conceivable that the antisera used were of insufficient potency. They were effective, however, in neutralizing all free virus in the media. Furthermore, fresh serum was added at the times of medium renewal or reseeding of the cells so that the ratio of antibody to residual virus increased considerably in the course of treatment of the cultures. In spite of these efforts, small amounts of virus apparently remained in the cells, where they escaped neutralization by antibodies. When the antisera were removed the infection presumably spread again throughout the cultures from the remaining infected cells. It is unlikely that during the period of serum treatment new cells became infected by extracellular spread of virus unless a certain proportion always remained unneutralized (34). The possibility must be considered that infection may be passed from mother to daughter cells, if such cells still divide, or that infection may be spread from one cell to another by close contact. This problem remains to be clarified.

No evidence was found which would indicate a selection of cells in persistently infected cultures. It is true that a large initial inoculum, providing several virus particles per cell, may cause some cytopathic effects. With smaller doses such effects may not be recognized. On the other hand, excessive inocula possibly may exert toxic effects as seen with thoroughly chick embryo–adapted allantoic strains of mumps virus, which are incapable of any multiplication in MCN or Lung-To cultures and thus fail to establish persistent infections (35). An answer was sought by studying clones derived from uninfected and persistently infected cultures. Of 50 cloned MCN cell lines none were destroyed by NDV and all became persistently infected. None were initially resistant to VSV but all developed resistance after persistent NDV infection was established. The 39 clones derived from MCN_{NDV} cultures were free of NDV, fully susceptible to VSV, and all acquired persistent infection on re-exposure to NDV. These data failed to reveal inhomogeneity of the MCN population or a selection of different cell types in persistently infected cultures. The fact that none of the cloned MCN_{NDV} lines carried NDV may be ascribed to the use of specific anti-viral serum in the cloning procedure, suggesting that under these conditions cures are more readily achieved than in the serum treatment of whole cultures.

With respect to the virus definite changes became apparent. The NDV strain derived from persistently infected MCN cultures produced only small, hazy plaques of about 1 mm. diameter on monolayers of chick embryo fibroblasts. The chick embryo–adapted strain, on the other hand, yielded mainly

large plaques, 2 to 3 mm. in diameter, and only an occasional small one. Such differences in plaque morphology have been noted also by Granoff (36). The HeLa cell–adapted virus gave intermediary results. These findings reflect most likely a selection of virus particles in tissue culture. Following back passage to the allantois of the chick embryo the virus obtained in allantoic fluid again produced small plaques, but since no serial passages were carried out, the stability of this property has not been ascertained. The change in activity had no effect upon the cytopathogenicity of the virus for MCN cells. While some lesions were noted on first passage with the larger inocula no cytopathic effects were seen on subsequent serial transfers. However, as will be shown in a subsequent paper (33) the virus derived from persistently infected cultures established the carrier state, and with it resistance to VSV in MCN cultures more readily than the chick embryo–adapted virus.

SUMMARY

Inoculation of the MCN and Lung-To lines of human cells in continuous culture with Newcastle disease (NDV), mumps, or 6-6 viruses led to slight cytopathic effects (CPE) if the multiplicity of infection exceeded one. On second passage or with smaller initial inocula no CPE became apparent. The viruses multiplied, however, as determined by titrations in HeLa cultures or chick embryos. Indeed, persistently infected sublines of MCN and Lung-To were readily established without resort to special manipulations and some of these have been carried now for over 18 months on the same media and schedules as the uninfected parent strains.

The viruses were found to be associated mainly with the cells and only 1, or at most 10 per cent of it was detectable in the media. The titers obtained were always low in relation to the available cell population.

Reduction or even omission of the horse serum component in the media or ultraviolet irradiation of the cultures did not increase the yield of virus, and CPE became apparent only when similarly treated, uninfected cultures were, likewise, affected by the manipulations.

The persistently infected cultures differed from their uninfected counterparts in that they exhibited (*a*) decreased cellular growth rates and ultimate yields; (*b*) increased aerobic glycolysis; and (*c*) a high degree of resistance to cytopathogenic viruses, influenza A (PR8), herpes simplex and, especially vesicular stomatitis (VSV) viruses.

Prolonged treatment of persistently infected cultures by addition of specific antiviral immune sera to the media reduced significantly the amount of virus present and the degree of resistance to VSV. However, upon removal of the sera after as many as 187 days of treatment the viruses reappeared in all but one instance. The cured culture, on reinfection, became again persistently infected.

No evidence was obtained to indicate genetic inhomogeneity of the cell

populations. Of 50 cloned MCN lines none was destroyed by NDV and all became persistently infected. None were initially resistant to VSV but all after establishment of persistent NDV infection. All 39 cloned lines derived from MCN_{NDV} cultures in the presence of anti-NDV serum, were free of virus and susceptible to VSV, and all acquired persistent infections and with it resistance to VSV following inoculation of NDV.

NDV maintained in MCN cultures differed from the parent, chick embryo–adapted strain with respect to its plaque morphology. Whereas the former yielded only plaques on monolayers of chick embryo fibroblasts which were of pin-point size and hazy, those obtained with the latter were rarely of this type and mostly large and clear. This apparent selection of virus particles did not alter significantly their behavior with respect to cytopathogenicity for uninfected MCN cultures.

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