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Studies on Persistent Infections of Tissue Culture

VI. Reversible Changes in Newcastle Disease Virus Populations as a Result of Passage in L Cells or Chick Embryos

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Populations of the Victoria strain of Newcastle disease virus (NDV), reisolated from persistently infected L-cell cultures and passed twice in the embryonated hen's egg ($NDV_{L\text{-}E\text{-}2}$), were found to differ strikingly from the original, chick embryo-adapted virus (NDV_O). After exposure of L cells to NDV_O at high multiplicities of infection, all cells became abortively infected; they produced only small aggregates of viral antigen and few, if any, infectious virus particles, but they yielded large amounts of interferon. No cytopathic effects (CPE) were noted, and the cultures survived readily as viral carriers. In contrast, $NDV_{L\text{-}E\text{-}2}$ yielded under similar conditions large quantities of viral antigen and infectious virus particles, but no detectable interferon, and the cultures were rapidly destroyed. This change in "virulence" was at least partially reversible by further serial passages of $NDV_{L\text{-}E\text{-}2}$ in chick embryos, as was evident from a consecutive decrease in CPE with a concomitant increasingly rapid recovery of the L-cell cultures, gradually diminishing yields of infectious viral progeny, and the returning of a capacity to induce interferon synthesis. Thus, $NDV_{L\text{-}E\text{-}16}$ resembled NDV_O in many aspects, except for a less striking reduction in its ability to replicate in L cells. Although a selection of viral variants under the given sets of conditions has not been entirely excluded, the establishment of "avirulence" appears to be largely explained by a gradual accumulation of noninfectious, interferon-inducing components in the course of serial passages in the embryonated hen's egg, and the acquisition of "virulence" by a loss of these components. The evidence is as follows. (i) By a step-wise decrease in the dose of virus and restriction of the analyses to the first infectious cycle, a multiplicity of infection was ultimately reached for all "avirulent" populations at which infected cells produced normal yields of infectious viral progeny; i.e., the interferon-inducing components were diluted to noneffective levels. The lowest multiplicity which resulted in a measurable reduction in infectious virus replication was also the last one to induce detectable interferon synthesis. (ii) All viral clones derived from "avirulent" populations behaved like $NDV_{L\text{-}E\text{-}2}$ rather than like the parent viral suspensions, except that some of them elicited small amounts of interferon in L cells. The interferon-inducing components were reduced or lost in the cloning procedures. The nature of the interferon-inducing components has not been established. These components, which were neutralized by rabbit sera against "virulent" $NDV_{L\text{-}E\text{-}2}$ populations, may represent largely inactive or incomplete virus particles; however, the infectious virus-hemagglutinin ratios of "avirulent" populations were mostly of an order similar to those of "virulent" populations. The interferon-inducing components aborted the infectious process in cells simultaneously invaded by infectious virus particles. The implications of these findings are discussed.

In a previous study (13), factors responsible for the establishment and maintenance of persistent Newcastle disease virus (NDV) infections in a subline of Earle's strain L cells were analyzed. For these experiments, virus was employed which

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had undergone two to four allantoic passages after reisolation in chick embryos from L-cell carrier cultures. Exposure of L cells to such virus preparations (now referred to as $NDV_{L\text{-}E\text{-}2}$, $NDV_{L\text{-}E\text{-}3}$, etc., depending upon the number of egg passages) at an input multiplicity of infection of 10 or greater gave the following results: (i) there was a pronounced cytopathic effect (CPE) and the cultures died; (ii) all cells contained, within 20 to 24 hr, large amounts of viral antigen, as was evident from specific immunofluorescence throughout the cytoplasm; (iii) infected cells yielded large numbers of plaque-forming units (PFU) and in addition about five times as many noninfectious, hemagglutinating (HA) virus particles, as determined by the PFU-HA ratios of the viral progeny; and (iv) little, if any, interferon was produced. These observations differed markedly from previous results obtained with the original strain of NDV (now designated NDV_O) prior to passage through L-cell cultures (1, 8, 9). In the earlier experiments, exposure of L cells to NDV_O at high multiplicity induced little or no CPE, the cultures readily survived, and considerable amounts of interferon were produced within 24 hr. A similar degree of resistance of L cells to NDV was reported by others (11, 18).

The purpose of the present study was to confirm the apparent difference between the two types of virus preparations in simultaneous tests under strictly comparable conditions. An evaluation of factors responsible for the difference in behavior between the virus populations is presented.

MATERIALS AND METHODS

The cells used, the relevant methods for immunofluorescence, hemagglutination, infectious virus, and interferon assays have been described (8, 13).

Viruses. The various preparations of the Victoria strain of NDV were obtained as follows.

NDV_L was reisolated in embryonated eggs from L-cell carrier cultures 2 years after their initiation with NDV_O. Cells from a 500-ml Blake bottle culture were scraped into 25 ml of medium (60% medium 199, 30% Scherer's maintenance medium, and 10% inactivated horse serum) and dispersed by vigorous pipetting. Volumes of 0.5 ml of this suspension were inoculated into the allantoic cavity of 11-day-old chick embryos. After 48 hr of incubation at 37 C, the eggs were cooled at 4 C, and the allantoic fluids were collected individually and tested for HA activity. Positive fluids were pooled, clarified at $450 \times g$ for 10 min, and distributed in ampoules which were stored in a Dry Ice chest. This preparation ($NDV_{L\text{-}E\text{-}1}$) contained 1.9×10^8 PFU and $1.3 \times 10_2$ HA units (HAU) per ml.

Further chick embryo passages were made by inoculation of 0.5-ml amounts of the preceding passages diluted to contain approximately 10^4 PFU per dose. The harvests of uneven-numbered passages were handled as described for $NDV_{L\text{-}E\text{-}1}$. The pooled allantoic fluids of the even-numbered passages were centrifuged at $60{,}000 \times g$ for 1 hr; the pellets were resuspended in medium to the original volume and were resedimented by the same centrifugal force. The final pellets were taken up in 0.1 volume of medium and were stored as described. These preparations ($NDV_{L\text{-}E\text{-}2}$, $NDV_{L\text{-}E\text{-}4}$, etc.) contained about 10 times as many PFU and HAU per milliliter as did the uneven-numbered preparations. The PFU-HA ratios were all of the same order as that of $NDV_{L\text{-}E\text{-}1}$.

NDV_O was concentrated from infected allantoic fluids by the procedures described for $NDV_{L\text{-}E\text{-}2}$. The final preparation contained 3.4×10^8 PFU and 6.4×10^2 HAU per ml and a PFU-HA ratio of 0.5×10^6.

Many of the virus preparations were titrated also in the allantoic cavity of chick embryos. The titers were generally slightly higher than those obtained by plaque assay, but they revealed no systematic differences referable to the passage history of the virus.

Virus clones obtained by plaque passages. Isolated plaques on monolayers of chick embryo fibroblasts were picked for four serial passages. Materials from individual plaques of the fourth passages, containing between 10 and 1,000 PFU were inoculated into 11-day-old chick embryos. Virus stocks were prepared from the second allantoic passage according to the techniques described for $NDV_{L\text{-}E\text{-}2}$.

Virus clones obtained by serial limiting dilution passages. Serial threefold dilutions in the vicinity of the infectivity end points were prepared from the various virus preparations, and were inoculated into eight chick embryos each. The allantoic fluids were collected individually 48 hr later and were tested for the presence of HA activity. At the end point of the titration, usually only one or two allantoic fluids gave positive results, and these were selected for passage. Virus stocks were prepared from the fourth limiting dilution passage by the procedures used for $NDV_{L\text{-}E\text{-}2}$.

Exposure of L cells to virus. Volumes of 25 ml of L-cell suspensions containing approximately 5×10^5 cells per milliliter were exposed to virus preparations at known input multiplicities and were incubated with agitation at 37 C for 1 hr. At the end of the adsorption period, the suspensions were centrifuged at $100 \times g$ for 5 min at 24 C. The sedimented cells were washed three times in medium, the first and second times with the addition of anti-NDV serum (1:25) to neutralize remaining extracellular virus. The cells were then suspended in medium, counted, adjusted to 10^5 cells per milliliter, and seeded into culture tubes with or without cover slips. The cover slips were harvested 20 hr later and stained with fluorescein-conjugated anti-NDV_O rabbit globulin to determine both the percentage of infected cells and the relative amount of antigen in the cells. At various times after infection, cells from tubes without cover slips were scraped into the medium, pooled, and counted. Samples of the pool were stored at −20 C for plaque titrations on chick embryo fibroblasts and interferon assays on monolayers of L

TABLE 1. *Exposure of L cells to $NDV_{L\text{-}E\text{-}2}$ and NDV_O*

Virus	Input multiplicity	Maximal degree of CPE	Fate of culture	Immunofluorescence		PFU/infected cell		Interferon units/10^5 cells at 20 hr
				Cells stained at 20 hr	Amt of antigen/cell	4 hr	20 hr	
				%				
$NDV_{L\text{-}E\text{-}2}$	20	4+	Loss	100	Large	0.01	200	<3
NDV_O	2.9	0	Survival	91	Small	0.01	0.002	30

cells. Additional tubes were kept for observation of the degree of cytopathic effects and to determine whether the cultures would eventually die or survive as viral carriers.

Other methods are described under Results.

RESULTS

Exposure of L cells to virus populations at high multiplicities of infection. Previous experiments indicated that remarkable differences exist between the original, chick embryo-adapted Victoria strain of NDV (NDV_O) and the same strain of virus reisolated from chronically infected L-cell cultures and passed twice in embryonated eggs ($NDV_{L\text{-}E\text{-}2}$). This was firmly established by the first experiment to be presented, in which both types of virus populations were used to infect L cells under strictly comparable conditions. As shown in Table 1, exposure of L cells to $NDV_{L\text{-}E\text{-}2}$ at an input multiplicity of 20 resulted in severe CPE and loss of the cultures; production of considerable amounts of viral antigen in every cell, as evident from diffuse immunofluorescent staining (*see* Fig. 1b of reference 13); replication of large numbers of infectious virus particles per infected cell; and little, if any synthesis of interferon. Conversely, exposure of L cells to NDV_O at an input multiplicity of 2.9 resulted in no CPE and survival of the cultures; formation of only small aggregates of viral antigen in individual cells (*see* Fig 1c of reference 13); production of few, if any, infectious virus particles per infected cell; and synthesis of considerable amounts of interferon.

Some of the above L-cell cultures exposed to NDV_O were maintained further by periodic refeeding followed by subcultivation in 500-ml Blake bottles. At 90 days after inoculation, the presence of a persistent infection was ascertained by challenge of the cultures with vesicular stomatitis virus (VSV) at an input multiplicity of 0.1. Control cultures were completely destroyed within 3 days after the addition of VSV, whereas the cell populations previously exposed to NDV_O were resistant because of interference. Parallel carrier cultures were used to reisolate the virus in a manner identical to that described for $NDV_{L\text{-}E\text{-}2}$. This preparation, $NDV_{O\text{-}L\text{-}E\text{-}2}$, contained 3.1×10^3 HAU and 5.2×10^9 PFU per ml of suspension. Exposure of cells to this seed at an input multiplicity of 25 again resulted in a high degree of CPE, but the cultures ultimately survived. All the cells were stainable within 24 hr by fluorescent anti-NDV_O globulin, and most contained large amounts of antigen. An average yield of 75 PFU of NDV per stainable cell and less than 3 units of interferon per 10^5 cells were produced.

Selection of an NDV population resembling NDV_O by serial passage of $NDV_{L\text{-}E\text{-}2}$ in eggs. To determine whether the change in properties of the viral populations was reversible, $NDV_{L\text{-}E\text{-}2}$ was subjected to further serial passages in chick embryos, with approximately 10^4 PFU as inoculum each time. Seeds were prepared according to the procedure described for $NDV_{L\text{-}E\text{-}1}$ for the odd-numbered passages, or according to the one described for $NDV_{L\text{-}E\text{-}2}$ for the even-numbered passages (see Materials and Methods).

The concentration procedure employed in the preparation of the even-numbered seeds did not appear to result in loss of either hemagglutinating activity or infectivity, and no significant differences were found among the various preparations with respect to their PFU-HA or ID_{50}-HA ratios.

Three separate experiments were performed to evaluate the behavior of these various seeds in L cells at high input multiplicities of infection. All of the virus preparations elicited the production of large amounts of stainable viral antigen in most of the infected cells. Other results are presented in Table 2 and Fig. 1.

Table 2 shows that serial passage of NDV_L in eggs gradually resulted in virus populations which, unlike $NDV_{L\text{-}E\text{-}2}$, caused only slight cytopathic effects with ready survival of the cultures, yielded little infectious virus progeny, and induced the synthesis of large amounts of interferon within 24 hr. Up to the fourth egg passage ($NDV_{L\text{-}E\text{-}4}$), the virus populations behaved much like $NDV_{L\text{-}E\text{-}2}$. In contrast, $NDV_{L\text{-}E\text{-}16}$ behaved in many respects like NDV_O, except that it produced more stainable antigen and larger numbers of

TABLE 2. *Exposure of L cells to serial egg passage seeds derived fom* NDV_L

Expt no.	Inoculum	Input multiplicity	Maximal degree of CPE	Fate of cultures	PFU/ infected cell (20 hr)	Interferon units/ 10^5 cells (20 hr)
1	$NDV_{L\text{-}E\text{-}4}$	13	4+	Loss	130	<3
	$NDV_{L\text{-}E\text{-}6}$	13	3	Survival	130	<3
	$NDV_{L\text{-}E\text{-}8}$	17	2	Survival	83	18
2	$NDV_{L\text{-}E\text{-}10}$	14	1	Survival	20	24
	$NDV_{L\text{-}E\text{-}12}$	12	1	Survival	14	23
	$NDV_{L\text{-}E\text{-}14}$	8	0	Survival	10	31
	$NDV_{L\text{-}E\text{-}16}$	13	0	Survival	9	23
3	$NDV_{L\text{-}E\text{-}4}$	11	4+	Loss	50	<4
	$NDV_{L\text{-}E\text{-}6}$	10	3	Survival	30	7
	$NDV_{L\text{-}E\text{-}7}$	7	2	Survival	25	15
	$NDV_{L\text{-}E\text{-}8}$	13	2	Survival	28	16
	$NDV_{L\text{-}E\text{-}10}$	11	1	Survival	15	30
	$NDV_{L\text{-}E\text{-}14}$	12	0	Survival	6	32
	$NDV_{L\text{-}E\text{-}16}$	11	0	Survival	8	32

infectious virus particles per infected cell than did the parent virus population.

Figure 1 presents the combined data from the three experiments with respect to cytopathic effects and virus and interferon production. It is shown (i) that serial egg passages of NDV_L did lead to virus populations with a gradually decreasing capacity to produce cytopathic effects and infectious virus and with a gradually increasing ability to induce synthesis of interferon, and (ii) that certain intermediate populations ($NDV_{L\text{-}E\text{-}7}$ and $NDV_{L\text{-}E\text{-}8}$) elicited the production of considerable amounts of both infectious virus and interferon. All surviving L-cell cultures remained persistently infected. Virus populations reisolated from ensuing carrier cultures, e.g., those exposed 3 months previously to $NDV_{L\text{-}E\text{-}16}$, exhibited again the properties of $NDV_{L\text{-}E\text{-}2}$.

It became apparent that the extent of cytopathogenicity, or "virulence," of a virus population for L cells is grossly paralleled by its ability to produce infectious virus progeny and inversely related to its capacity to induce interferon synthesis. An avirulent virus population (NDV_O or $NDV_{L\text{-}E\text{-}16}$) could be converted at will to virulence during chronic infection of L cells (e.g., $NDV_{L\text{-}E\text{-}2}$), and this conversion was reversible by serial passages in chick embryos.

Exposure of L cells to various NDV populations at decreasing input multiplicities. The gain or loss in virulence of NDV for L cells could reflect (i) a selection of mutants which gives rise to either dominantly virulent or dominantly avirulent virus populations, (ii) a change in the relative frequencies of at least two different components in the virus populations, or (iii) a combination of

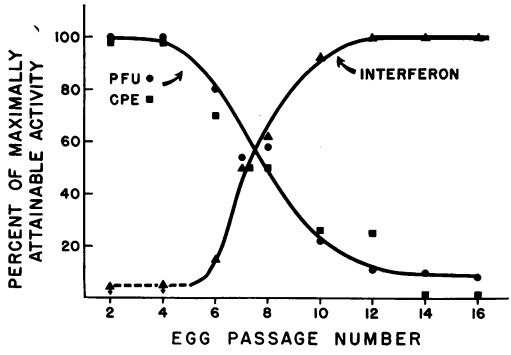

FIG. 1. *Changes in* NDV_L *populations on serial passage in chick embryos as evident from exposure of L cells at high multiplicities of infection.*

these two. To test these possibilities, L cells were exposed to various types of virus preparations at decreasing input multiplicities or to cloned virus populations derived from virulent or avirulent parent stocks. Results of the first approach will be presented below, and those of the second type of experiment, in the next section.

If an avirulent virus preparation were composed almost exclusively of avirulent particles, infection of L cells at low input multiplicities should yield in the first reproductive cycle [24 hr (13)] the same type of results obtained after a saturation inoculum on the basis of yields per infected cell. If, however, avirulence were due to components in the population which prevent or reduce replication of any virulent virus particles that might also be present, a decrease in the multiplicity of infection should eventually reduce the concentration of the interfering entities to ineffective levels

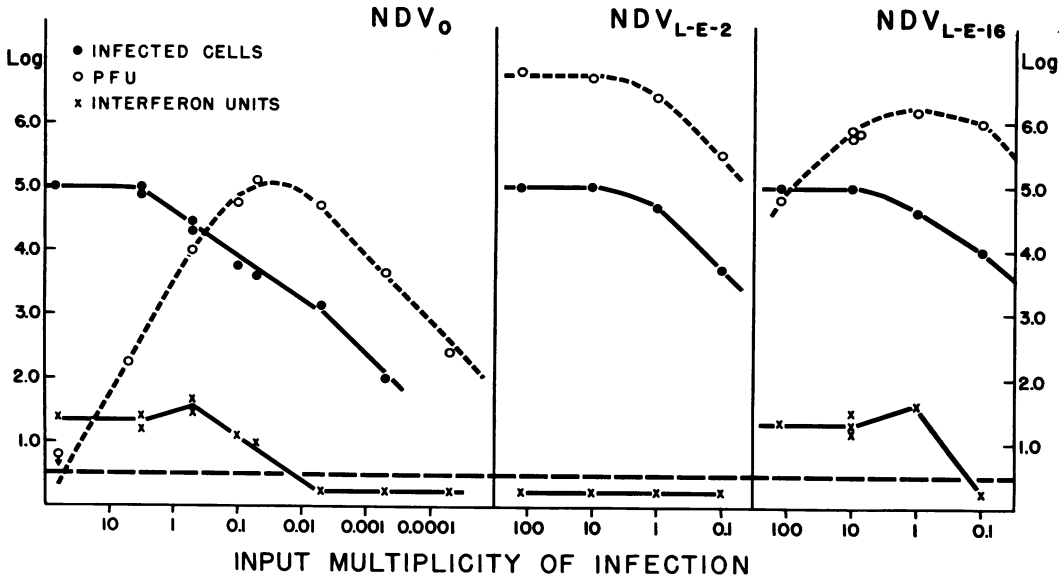

FIG. 2. *Exposure of L cells to NDV$_0$, NDV$_{L\text{-}E\text{-}2}$, and NDV$_{L\text{-}E\text{-}16}$ at different multiplicities of infection. Number of immunofluorescent cells, and yields of PFU and interferon per 10^5 total cells.*

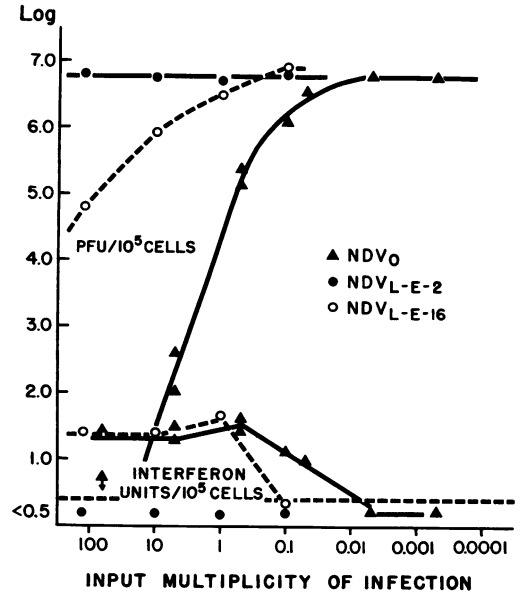

FIG. 3. *Exposure of L cells to NDV$_0$, NDV$_{L\text{-}E\text{-}2}$, and NDV$_{L\text{-}E\text{-}16}$ at different multiplicities of infection. Comparison of yields of PFU per 10^5 immunofluorescent cells and relation to inteferon production.*

and permit the virulent virus particles to produce normal yields of viral progeny. The first multiplicity which would permit unhampered replication of the virulent particles would provide a basis for rough estimates of the concentration of interfering units in avirulent virus populations.

In accordance with the above considerations, L cells were exposed by the usual technique to NDV$_0$, NDV$_{L\text{-}E\text{-}2}$, and NDV$_{L\text{-}E\text{-}16}$ at various multiplicities. The cultures were assayed 4 and 20 hr later for cell numbers, infectious virus concentrations, and interferon titers. Cover slip cultures were set up in parallel to determine the number of infected cells by virus-specific immunofluorescence. At 4 hr after infection, neither interferon nor stainable cells were detectable in any of the cultures. The numbers of PFU obtained at this time ranged from slightly more than 10^3 to none, depending upon the input multiplicity, and represented most likely input virus. The results obtained at 20 hr from a series of experiments are combined and summarized in Fig. 2 and 3.

With the virulent virus population (NDV$_{L\text{-}E\text{-}2}$), the number of infected (staining) cells conformed to expectations based upon the input dose of virus (Fig. 2). The total yields of infectious viral progeny were very similar when the cells were exposed at an input multiplicity of 10 or 200. The yields of PFU per infected cell were constant over the whole range of multiplicities employed, and amounted to an average of about 60 (Fig. 3). No trace of interferon could be detected in any of the first-cycle yields. If second cycles were permitted to take place, interferon production became

TABLE 3. *Infectivity and hemagglutinin titers of parent and cloned viral populations*

Virus population	Method of prepn	Plaque morphology[a]	PFU	HA	PFU/HA
NDV_O (avirulent)					
Parent	—	L, 60%; S, 40%	3.4×10^8	6.4×10^2	5.3×10^5
Clone A	Plaque (4×)	L	1.6×10^9	1.3×10^3	1.2×10^6
Clone B	Plaque (4×)	S	8.0×10^9	1.3×10^3	6.1×10^6
Clone C	Limiting dilution (4×)	—	6.1×10^9	1.3×10^3	4.7×10^6
$NDV_{L\text{-}E\text{-}2}$ (virulent)					
Parent	—		2.3×10^9	1.3×10^3	1.8×10^6
Clone A	Plaque (4×)	L	1.6×10^9	1.3×10^3	1.2×10^6
$NDV_{L\text{-}E\text{-}16}$					
Parent	—	L, 50%; Lr, 50%	1.9×10^9	1.3×10^3	1.4×10^6
Clone A	Plaque (4×)	L	9.4×10^9	1.3×10^3	3.4×10^6
Clone B	Plaque (4×)	Lr	4.1×10^9	2.6×10^3	1.6×10^6
Clone C	Limiting dilution (4×)	—	4.2×10^9	1.3×10^3	3.2×10^6

[a] L = large, 3 mm, smooth-edged; S = small, 1 mm, smooth-edged; Lr = large, rough-edged.

detectable at multiplicities of infection below, but not above, 5.

The results obtained with NDV_O (avirulent) were strikingly different. At an input multiplicity of 5 or greater, practically all cells became infected and produced some viral antigen (Fig. 2). As the dose of virus was decreased, the decline in the number of infected cells was initially not as steep as expected, but assumed the proper slope when the input multiplicities were reduced to 0.005 or less. This effect may denote the presence in the NDV_O population of a factor which completely protected some of the L cells against infection. The highest *total yields of PFU* were obtained at an input multiplicity of 0.01 to 0.05. These were about 50-fold lower than the maximal titers produced by the virulent $NDV_{L\text{-}E\text{-}2}$ populations. As the inoculum of NDV_O was increased, the yields of infectious virus declined steeply. The numbers of PFU found 20 hr after infection at an input multiplicity of 5 or greater were in fact smaller than those found at 4 hr, indicating that no infectious viral progeny was produced under these conditions. The *yields of PFU per infected cell* (Fig. 3) were normal when the input multiplicity of NDV_O was 0.005 or less, amounting to about 60 per cell. At increasingly higher input doses of virus, the yields per infected cell declined rapidly, and ultimately few, if any, of the cells produced infectious virus. Interferon production became detectable at an input multiplicity of 0.05, it reached a peak at an input multiplicity of 0.5, and then it assumed a plateau at a slightly lower level as the inoculum was further increased. The first appearance of interferon in the ascending order of multiplicities coincided with the first evidence for a slight reduction in viral progeny.

The results obtained with $NDV_{L\text{-}E\text{-}16}$ showed a less striking reduction in infectious progeny at high multiplicities of infection than did those produced by NDV_O. Total yields reached a peak at an input multiplicity of 1 (Fig. 2), which was only fourfold lower than the maximal titer obtained with $NDV_{L\text{-}E\text{-}2}$. At this level of infection, the number of PFU per infected cell was only slightly smaller than the normal yields observed with one-tenth the input dose (Fig. 3). Interferon production became detectable and reached a peak at an input multiplicity of 1, the first multiplicity at which virus production was detectably reduced. As with NDV_O, interferon titers reached a slightly lower plateau with the larger inocula.

Exposure of L cells to viral clones derived from virulent and avirulent populations of NDV. The above experiments indicated that NDV_O and $NDV_{L\text{-}E\text{-}16}$, rather than consisting entirely of avirulent infectious units, were composed of both virulent virus particles and avirulent entities capable of interfering with the replication of infectious virus. This was substantiated by a study of cloned virus populations derived from virulent $NDV_{L\text{-}E\text{-}2}$ as well as from avirulent NDV_O and $NDV_{L\text{-}E\text{-}16}$ stocks. The clones were obtained by four serial passages of picked plaques or by the limiting dilution technique, as described under Materials and Methods. The infectivity and hemagglutinin titers of the cloned seeds are shown in Table 3. L cells were exposed to these virus preparations at an input multiplicity of 10 or greater, and the results were assayed 20 hr later by the usual techniques. Some culture tubes were maintained longer to determine the ultimate fate of the cultures. The clone derived from $NDV_{L\text{-}E\text{-}2}$ behaved like the parent population

TABLE 4. *Exposure of L cells to viral clones derived from virulent and avirulent populations*

Parent population	Cloned virus prepn employed	Method	Input multiplicity	Maximal degree of CPE	Fate of cultures	PFU/infected cell (20 hr)	Interferon units per 10^5 cells (20 hr)
$NDV_{L\text{-}E\text{-}2}$ (virulent)	$NDV_{L\text{-}E\text{-}2}$ A	Plaque purified	23	4	Loss	102	<5
NDV_O (avirulent)	NDV_O A	Plaque purified	23	2	Survival	49	5
	NDV_O B	Plaque purified	15	1	Survival	47	11
	NDV_O C	Limiting dilution	15	3	Survival	27	7
$NDV_{L\text{-}E\text{-}16}$ (avirulent)	$NDV_{L\text{-}E\text{-}16}$ A	Plaque purified	13	2	Survival	105	8
	$NDV_{L\text{-}E\text{-}16}$ B	Plaque purified	10	3	Survival	81	10
	$NDV_{L\text{-}E\text{-}16}$ C	Limiting dilution	16	2	Survival	86	<5

(Table 4). It yielded large numbers of PFU per infected cell but no interferon, and the culture was destroyed. The clones derived from the avirulent populations differed in their behavior from those of the parent populations. The cytopathic effects initially were severe but the cultures recovered and survived as viral carriers. The infected cells contained at 20 hr large amounts of viral antigen, and the yields of PFU per infected cell were within the normal range or only slightly below. Most clones from the avirulent lines yielded detectable levels of interferon, but these amounted to less than 30% of the amounts produced by the parent populations.

DISCUSSION

The experiments described have shown marked differences among virus populations derived from the Victoria strain of NDV with respect to virulence for L cells when these were exposed at high multiplicities. Under these conditions of infection, NDV_O, the original virus maintained by uninterrupted chick embryo passages, may be termed avirulent, since the cultures showed limited CPE and readily survived. Although nearly all cells contained viral antigen within 24 hr, this was restricted to small aggregates in the cytoplasm. Little, if any, infectious viral progeny was produced, but considerable quantities of interferon were synthesized. In contrast, $NDV_{L\text{-}E\text{-}2}$, reisolated from L_{NDV} carrier cultures and passed twice in chick embryos, had acquired virulence for L cells in the course of the chronic infection. All cells infected by this virus produced large amounts of viral antigen in 24 hr, which was spread throughout the cytoplasm, and yielded numerous PFU of viral progeny, but synthesized no interferon. These changes were at least partially reversible by 14 additional passages of $NDV_{L\text{-}E\text{-}2}$ in chick embryos. Exposure of L cells to saturation inocula of $NDV_{L\text{-}E\text{-}16}$ permitted the cultures to survive and to synthesize high titers of interferon, but the production of viral antigen and of infectious progeny, although reduced, was greater than that observed with NDV_O. It is likely that further egg passages beyond $NDV_{L\text{-}E\text{-}16}$ would ultimately have led to virus populations in every aspect like NDV_O. The recorded changes apparently can be obtained at will either by passing virulent virus in chick embryos or by initiation of chronic infections with avirulent virus in L-cell cultures.

The results clearly show that virulent virus populations fail to induce significant interferon synthesis, whereas avirulent ones are capable of doing so. Such an inverse relationship between virulence and interferon production has been observed previously with measles (3), poliomyelitis (10), vaccinia (4), vesicular stomatitis (17), and other viruses (14, 15). This subject was reviewed extensively by Isaacs (10).

The observed changes in virulence were incompatible with host-induced modifications, reported also for NDV (2), because (i) they did not occur rapidly, and (ii) they were equally detectable also upon infection of chick embryo fibroblast cultures (V. ter Meulen and J. Rodriquez, *unpublished data*). The time required for conversion from avirulence to virulence in the course of chronic infections of L-cell cultures has not been studied in detail, but it was noted that virus reisolated 3 months after initiation of the carrier state failed to destroy all L cells exposed to it, whereas virus reisolated later did. The loss of virulence as a result of serial passages of NDV_L in chick embryos was clearly shown to be gradual. Even after 16 such passages ($NDV_{L\text{-}E\text{-}16}$), the virus still differed from NDV_O, as already discussed.

At an earlier passage level (e.g., $NDV_{L\text{-}E\text{-}7}$), the virus population induced in L cells production of substantial amounts of infectious virus as well

as of interferon. With the techniques used, it was impossible to determine clearly whether in this instance part of the cells yielded mainly infectious virus and the others mainly interferon, or whether individual cells produced both in considerable, although reduced, quantities. The fact that, with the 6th to 10th egg passage seeds of NDV_L, the yields of PFU were inversely proportional to those of interferon (Fig. 1) favors the first alternative. It is certain, however, that synthesis of large amounts of viral antigen in almost all of the cells does not per se prevent the cultures from yielding also considerable amounts of interferon, as is evident from results obtained with the chick embryo-passaged virus populations $NDV_{L\text{-}E\text{-}7}$ through $NDV_{L\text{-}E\text{-}16}$.

Most of the experiments were carried out with virus separated from allantoic fluid by two cycles of centrifugation. The observed changes in virulence were therefore most likely attributable to the viral population. Indeed, antisera prepared against the $NDV_{L\text{-}E\text{-}2}$ clone prevented production of interferon by NDV_O. Thus, the change could be due to a selection of mutants, or to variations in frequency of at least two viral components in the populations, or to a combination of both. Variants of NDV have been described which were obtained by cultural, physical, or chemical manipulations (5–7, 12, 18). Although this possibility has not been entirely excluded in the present studies, the available information points to changes in the frequency of two or more viral components as the major mechanism. By decreasing the doses of virus and restricting the observation to the first infectious cycle, a multiplicity of infection was eventually reached for all avirulent populations at which the infected cells produced standard yields of infectious progeny. With NDV_O this point was reached only at an input multiplicity of 0.005 and with $NDV_{L\text{-}E\text{-}16}$ at an input multiplicity of 0.1, when about 1 in 400 and 1 in 20 cells, respectively, became infected. These results suggest that the avirulent virus populations contained a component (or components) which interfered with replication of the infectious virus also present, and that about 20 times more of this component was present in NDV_O than in $NDV_{L\text{-}E\text{-}16}$. The results further indicate that it is this component which induced first-cycle interferon synthesis, since that multiplicity of infection in ascending order which provided first evidence of a decrease in viral yield per infected cell was also the one which induced the first detectable interferon synthesis. Assuming that the noninfectious components are solely responsible for interferon production, it may be calculated from the available data that NDV_O contained at least 40, and possibly more interfering particles for every infectious unit, and $NDV_{L\text{-}E\text{-}16}$ contained at least 2.

In accordance with the results obtained at low multiplicities of infection, it has not been surprising to find that viral clones derived from avirulent populations behaved like the virulent $NDV_{L\text{-}E\text{-}2}$, whether they were obtained by the limiting dilution technique in chick embryos or by picked plaque passages in chick embryo fibroblast cultures. All caused severe CPE and produced generally high yields of infectious virus per infected cell and only little, if any, interferon, but the cultures ultimately survived. Whether this difference from $NDV_{L\text{-}E\text{-}2}$ denotes that the cloned populations contained small amounts of the interfering components or that they represented viral variants cannot be decided at this time.

The nature of the interfering, interferon-inducing components is not yet established. At least part of them do not seem to be endowed with hemagglutinating activity. The PFU-HA ratios of the avirulent virus populations were either of the same order as those of virulent populations, or, at most, about 10-fold lower. Use of bovine, instead of chicken red cells for the hemagglutinin assays led to essentially similar results. Thus, thermally inactivated or incomplete NDV particles, both of which would be expected to cause hemagglutination, do not seem to account entirely for the observed results. It appears that the interfering components are not rapidly produced in detectable amounts on reisolation of virus from L_{NDV} carrier cultures, but that they accumulate gradually on continued serial passages in chick embryos. The accumulated components are evidently reduced or temporarily lost on cloning of avirulent virus populations. The interferon-inducing entities apparently abort the infectious process in cells simultaneously invaded by infectious virus particles. Although some viral antigen is synthesized under these conditions, little or no infectious virus is produced.

It would seem that NDV populations are avirulent for L-cell cultures when they contain noninfectious, interferon-inducing particles, and they are virulent in the absence of these components. As shown previously (13), infectious virus particles do not produce interferon in L cells, but the viral progeny obtained on exposure of L cells to $NDV_{L\text{-}E\text{-}2}$ contain noninfectious particles which in second and further cycles induce interferon synthesis and abort viral replication. The nature of the interfering components, as well as the applicability of these findings to avirulence in other host-virus systems, is being studied further.

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