SYMPOSIUM ON THE BIOLOGY OF CELLS MODIFIED BY VIRUSES OR ANTIGENS[1]

IV. Single-Cell Techniques in Tracing Virus-Host Interactions[2]

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INTRODUCTION

Study of bacteriophage has enjoyed two unique advantages over animal virology. On the one hand, bacterial virus particles could be precisely scored through the ability of single particles to create isolated lytic areas or plaques on layers of suitable host cells. Seven years ago this advantage yielded to the equivalent discovery in the animal virus field by Dulbecco (9). On the other hand, the host cells of bacteriophages also were susceptible to neat, quantitative titration by means of their ability to form discrete, countable, macroscopic colonies. With the development in our laboratory of quantitative colony-forming, plating methods for single mammalian cells (26, 34, 37), the second unique advantage of bacteriophage studies has also been secured for animal systems. Within a relatively short time, animal virology has progressed in the variety of host systems available, from the whole animal; to the egg and its several, isolable parts; to massive cell populations grown in tissue culture; to single cells. Here is presented a sampling of the kinds of problems amenable to attack in the course of tracing virus-host interactions with single-cell plating techniques.

The mushrooming importance of virus carrier and latent virus states in animal virology points out the need for increasingly finer tools of virus detection and quantitation. The study of virus-cell interaction at the single-cell level helps fill this need. Many workers have skillfully manipulated cell and virus at this level of attack and produced rewarding results.

The investigations of Cieciura (5) and Puck and Cieciura (33) in demonstrating the dual potential of a Newcastle disease virus-carrying HeLa cell provide such an example. With the precision of cell-plating and plaque-forming techniques, they have shown that, under appropriate conditions, a single cell may act as an infective center and produce plaques, or reproduce faithfully and form a countable colony.

The exciting work of Rubin and Temin (38, 43) illustrates the quantification available at the level of single tumor cells. Using single-cell plating, these workers quantitate the conversion of individual normal chick cells to the tumorous Rous sarcoma state. Although so far these experiments have utilized the principle of the feeder system (34), undoubtedly newer advances in cell nutrition soon will permit elimination of the feeder cells (36).

With the development of interest in various kinds of elusive viruses, including tumor agents, the need for better methods of virus detection has increased. The recent work we have carried out with Tolmach demonstrates the increased yield of certain viruses from X-ray-induced giant cells ((25) and *unpublished data*). The rapid rate at which these cells disintegrate under virus attack makes them especially useful detectors of virus action ((25) and S. Levine, *personal communication*).

The ready manipulation of single cells also makes possible quantitative studies of agents other than viruses that affect cell reproduction. As two recent examples may be cited the demonstration in our laboratory of the multihit nature of the survival curve of ultraviolet-irradiated HeLa cells (19), and the one-hit characteristic of diphtheria toxin lethality shown by Kaplan and Lennox (17). In the present paper, detailed account will be given of some specific problems pur-

[1] This symposium was held at the 59th Annual Meeting of the Society of American Bacteriologists in St. Louis, Missouri, on May 11, 1959, under the sponsorship of the Division of Medical Bacteriology, Immunology, and Virology, with Dr. R. Walter Schlesinger as convener.

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sued by the author at the level of single cell and virus particle interaction.

SINGLE-CELL PLATING TECHNIQUES

Virus interaction with single cells entails manipulation of host cell populations as suspensions of singly dispersed elements so as to preserve colony-forming ability for each isolated member. Briefly the following routine procedures are used to accomplish this end with S3 clonal strains of the HeLa line. Host cells are maintained in a cell farm as glass-attached monolayers. These are readily removed by a 10- to 15-min exposure to dilute trypsin or Versene. Mild pipetting then produces a uniform suspension of monodisperse cells. These suspensions may be counted in a hemocytometer, diluted, and plated in standard bacteriological fashion (26, 37), or plated directly and then accurately scored by counting individual cells observed through small windows drilled in metal discs. The perforated discs are affixed to the outside bottom of a petri dish and permit clear viewing of individual cells. The use of the window-counting technique makes possible virtual elimination of the sampling error (24). When plates inoculated with a dilution containing on the average 100 single cells are incubated for 8 to 9 days, fixed, and stained, one observes 100 $\pm$ 20 visible colonies. Throughout the various experimental manipulations described herein, control cells give this high plating efficiency—a tribute to the hardy nature of the S3 strain HeLa cell. Experimental procedures designed to extend single-cell plating techniques to cell types other than HeLa are available (26, 35, 36).

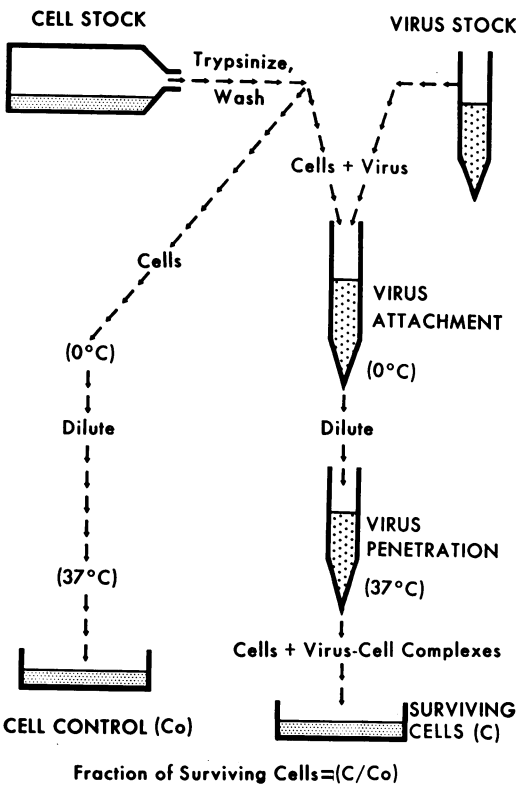
QUANTITATION OF CELL KILLING BY VIRUSES

A. Single-Cell Survival Procedure

The ability to determine accurately the number of virus particles of different kinds needed to destroy a cell's reproductive potential, and the precision with which this destruction can be measured, form the basis of experiments designed to probe virus-host cell interaction where the criterion of cell death is the measure of a virus particle's activity. Here, cell death is defined in the usual microbiological sense, *i.e.*, failure of a microorganism to reproduce and form a macroscopically visible colony under standard growth conditions. When populations of monodisperse HeLa cells are exposed to various multiplicities of virus and virus attachment and penetration is complete (as evidenced by the formation of a virus-cell complex, stable to dilution and to treatment with virucidal growth medium), these complexes are plated and the fraction of cells which did not adsorb a lethal amount of virus is scored as the fraction of cells which form visible colonies 8 to 9 days later. The plating must be done in a semisolid medium, or in one toxic to free but not to intracellular virus, so that cross infection of cells by newly synthesized virus will not occur. This so-called single-cell survival procedure is the basic experimental technique used to detect virus cell-killing activity, and is presented schematically in figure 1. Experimental details of this procedure have been presented elsewhere (22–24).

B. Kinetics of Cell Killing by Virus

When the kinetics of cell killing are studied as described, results of the type illustrated in figures 2 and 3 are obtained. Figure 2 consists of photographs of colonies which have developed after 11 days of incubation in 50-mm petri dishes seeded with HeLa cells exposed to different concentrations of Newcastle disease virus (NDV) as described above. It is clear that no ambiguity exists in counting survivors. The data in figure 3 tell us two things about the kinetics of cell killing by virus. First, the one-hit exponential nature of the survival curve reveals that the interaction of an individual HeLa cell with only 1 virus particle suffices to bring about cell death. Here, the fraction of cells escaping the lethal action of Newcastle disease virus is found to equal the term of the Poisson distribution formula for those cells which have escaped infection, namely, e^{-m}, where m is the average number of particles attached per cell, *i.e.*, the multiplicity. Theoretical curves for cases where the attachment of 2 and 3 particles per cell would be required for cell killing are shown for comparison. Secondly, the close agreement of experimental and theoretical 1-particle-to-kill survival curves over a 3-log range attests to the homogeneous susceptibility of 99.9 per cent of the members of this clonally derived HeLa cell strain.



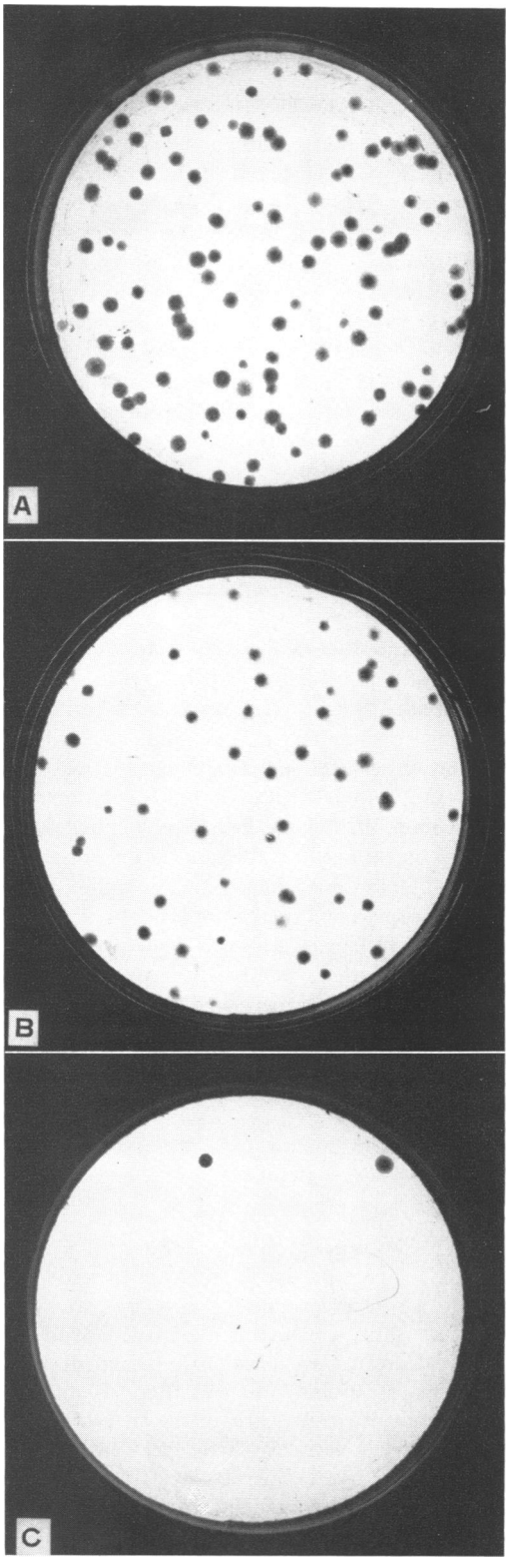


Figure 1. Schematic representation of the single-cell survival procedure. HeLa cells are trypsinized, washed, and centrifugally deposited in a tube to which an appropriate dilution of virus is added for formation of the initial virus-cell complex, usually at 0 to 2 C. Virus adsorption is terminated by diluting the complexes formed at low temperature into nonvirucidal medium at 37 C, where they are held until penetration is complete. Virus-treated cells are then counted, diluted, and plated in a virucidal complete growth medium. After 8 to 9 days of incubation, colonies developing on the plate are fixed, stained, and counted to score the fraction of surviving cells.

C. Assay and Properties of Cell-Killing Particles

The precise manner in which the cell-killing activity of a virus suspension may be measured permits an additional criterion by which virus activity can be accurately scored. For example, from figure 3 we can see that a certain virus concentration results in the death of 63 per cent of the cell population (37 per cent survivors). From the Poisson distribution, this represents, on the average, 1 cell-killing particle (ckp) attached per cell. In figure 3 this conversion has been applied,

and the equivalent cell-killing particle multiplicity (m_{ckp}) placed on the top abscissa. The additional knowledge of the concentration of host cells and of the fraction of virus adsorbed permits calculation of the total number of cell-killing particles in a given preparation. For example, determination of the absolute cell-killing particle multiplicity (m_{ckp}) is calculated from the fraction of cell survivors (C/C_0) and the Poisson distribution, where

$$e^{-m_{ckp}} = C/C_0.$$

Cell-killing particles (ckp) are then enumerated as follows:

$$\text{ckp} = \frac{m_{ckp}hd}{a}$$

where

ckp = concentration (particles per ml) of virus capable of killing cells;
h = host cell concentration (cells per ml) during virus attachment;
d = reciprocal of the virus dilution; and
a = fraction of total virus attached.

Typical cell-killing particle assays for two strains of Newcastle disease virus are presented in table 1. Plaque-forming titers are included for comparison. It is important to note that although the plaque-forming and cell-killing particle titers of the Beaudette strain are experimentally equal, plaque formation in the Vaccine strain is extremely labile and virtually disappears even when stocks are stored at −50 C for a few months, a condition which leaves the ckp numbers unaltered. This interesting characteristic is currently under investigation. More detailed comparisons of assayable virus properties have been presented elsewhere (23).

Although the stability of the cell-killing property resembles that of hemagglutination with respect to low temperature storage, its response

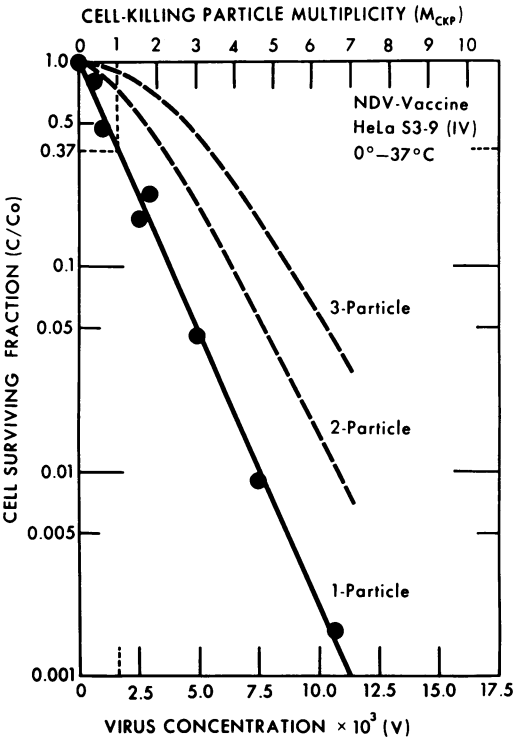


Figure 3. Survival curve of HeLa S3-9(IV) cells exposed to Newcastle disease virus (NDV) strain Vaccine. Suspensions of monodisperse HeLa cells were mixed with different amounts of NDV and plated in the standard manner, and the fraction of cell survivors determined from colony counts and plotted as a function of the virus concentration. Theoretical plots for 1-particle, 2-particle, and 3-particle mechanisms of kill are indicated. The three curves have been derived from the Poisson distribution

$$P_{(r)} = \frac{m^r e^{-m}}{r!}$$

where $P_{(r)}$ is the probability of the cells in a given population attaching r virus particles when the average multiplicity of infection is m. The fraction of cells escaping infection for the three plots are:

1-Particle curve—$P_{(0)} = e^{-m}$

2-Particle curve—$P_{(0)} + P_{(1)} = e^{-m}(1 + m)$

3-Particle curve—$P_{(0)} + P_{(1)} + P_{(2)} = e^{-m}(1 + m + m^2/2)$.

The cell-killing particle multiplicity (m_{ckp}) on the top abscissa was calculated from $C/C_0 = e^{-m_{ckp}}$. When the cell surviving fraction (C/C_0) = 0.37, then $m_{ckp} = 1.0$.

Figure 2. Colony formation by HeLa S3-9(IV) cells exposed to different concentrations of Newcastle disease virus (NDV), strain Vaccine. Photographs showing colonies formed on 50-mm petri dishes after inoculation with HeLa S3-9(IV) cells and incubation in a complete growth medium for 11 days at 37 C. Plate (*A*) received an inoculum of 100 cells with no NDV. Plates (*B*) and (*C*) were each seeded with 1000 cells which had attached, on the average, 3.2 and 6.4 NDV cell-killing particles, respectively.

TABLE 1

Typical cell-killing and plaque-forming particle assays of Newcastle disease virus strains Vaccine and Beaudette

Virus Strain	Stock of	Cell-Killing Particle (Titer per Ml)	Plaque-Forming Particle (Titer per Ml)
Vaccine	3/58	$8.0 \pm 0.8 \times 10^9$*	$<10^3$
	10/58	$7.2 \pm 1.5 \times 10^9$	5.1×10^4†
Beaudette	4/54	$1.9 \pm 0.3 \times 10^9$	2.1×10^9
	1/58	$2.3 \pm 0.4 \times 10^9$	1.9×10^9

* Mean and standard deviation of three independent assays (HeLa S3-9(IV) as host).

† Titers are the mean of plaque platings performed on chick embryo cell monolayers in triplicate using a modification of the Dulbecco plaque procedure (9) which scores the maximal number of NDV particles (23).

to ultraviolet or heat inactivation is quite different (24).

Here a certain uniqueness of cell-killing particle detection deserves elaboration. To detect a cell-killing particle, it is only necessary that the particle prevent cell reproduction. It is not necessary that the particle produce the successive waves of infection normally needed to score the plaque-forming, or disease-producing capacity of a virus. However, it should be pointed out that considerations of virus-attachment velocity limit accurate scoring of ckp to suspensions containing at least 10^5 particles per ml (24). The combined use of X-ray-induced giant HeLa cells as virus detectors (6, 25), and the window-counting technique reduce this value 2- to 3-fold (24).

We have determined, by examining individually hundreds of cells which have adsorbed a lethal dose of virus, that these cells do not divide even once provided of course that cell division has not already progressed before infection to the point where its completion is no longer preventable. Once past this point cells divide only once (24). Through use of the window-counting technique such cells are easily recognized and scored within 48 hr following virus attachment, thus permitting early detection and quantification of virus action.

PARAMETERS AFFECTING CELL KILLING BY VIRUS

With the afore-mentioned basic findings of cell killing by virus in mind, let us now consider some parameters that may affect the outcome of a virus-cell encounter, for example, virus multiplicity, temperature of attachment and penetration, pre- and post-treatment of the virus or of the virus-cell complex, and specific genetic characters in both the virus and the cell.

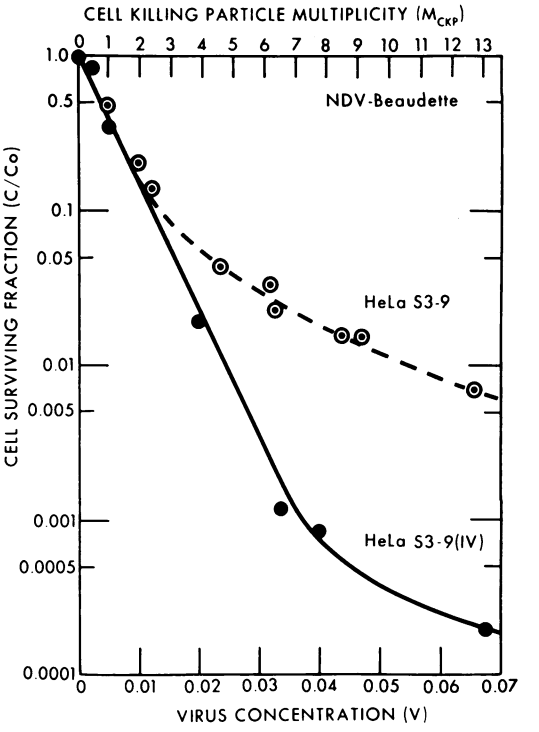


Figure 4. Survival curves of HeLa S3-9 and S3-9(IV) cells exposed to low multiplicities of Newcastle disease virus (NDV), strain Beaudette. Virus was attached separately to each cell type at 0 C and the resulting complexes were diluted, held at 37 C for 15 min, and plated and scored in the standard fashion.

A. The Cell Genome

Changes in the cell genome may have marked effects on virus cell killing. For example, a mutant subclone of HeLa S3-9 was isolated which displays more uniform response to the lethal action of NDV than does the parental cell strain. Figures 4 and 5 present survival curves of these two different lines of HeLa exposed separately to the Beaudette and Vaccine strains of NDV. It is important to note that both cell lines score the same number of killing particles, since the survival curves are exponential and superposable for low virus multiplicities. However, the curves for the parent strain, S3-9, begin to deviate from

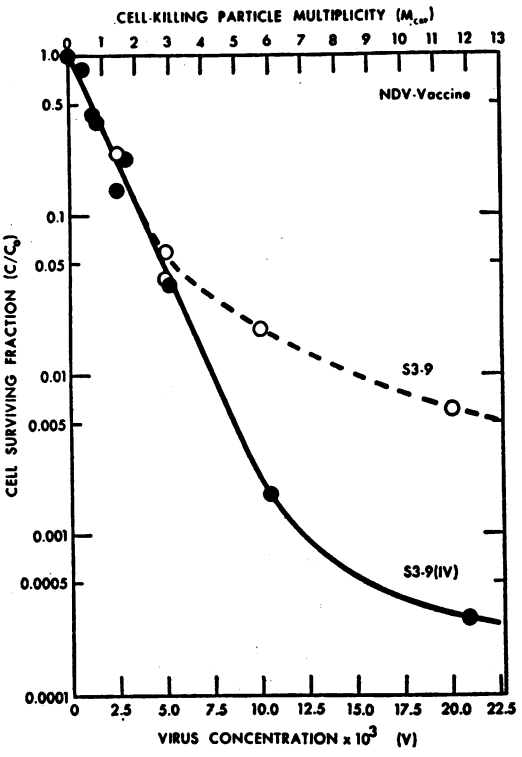


Figure 5. Survival curves of HeLa S3-9 and S3-9(IV) cells exposed to low multiplicities of Newcastle disease virus (NDV), strain Vaccine. Virus attachment, penetration, and cell plating conditions as in figure 4.

the 1-particle-to-kill relationship at ckp multiplicities greater than about 3, whereas those of the mutant line, S3-9(IV), obey the one-hit relationship until a multiplicity of about 6 has been reached, or a level of only 0.1 per cent survivors. Results similar to these have been obtained by Vogt with an S3 line of HeLa cells and poliovirus (44).

The reason for the different response to cell killing by these two mutants is still under investigation although it is evident that: (a) both mutants appear to be genetically stable, since over 60 generations of reproduction have not altered their behavior, (b) the kinetics of virus attachment to both cell lines is essentially identical, and (c) the fraction of virus particles destined to complete a lethal relationship with the cell is attained at essentially equally rapid rates on both cell mutants. For example, it was determined that the minimum possible surviving fraction for virus-cell complexes of either cell mutant is obtained within 5 min at 37 C, and that increasing the time to 1 hr did not increase the fraction of cells killed. Thus, at an attached multiplicity of 6 ckp, abortive penetration induced by the simultaneous addition of virus appears to be responsible for survival of the multiply infected cells in the case of S3-9 as host. This type of response recalls the behavior of the cell-associated NDV of Rubin *et al.* (39) during the latent period, the nonrecoverable and apparently noncell-killing NDV of Prince and Ginsberg (32), the "lost" poliovirus of Payne *et al.* (30), and the bound infectious virus (influenza) of Ackermann *et al.* (1). Further studies are required to elucidate abortive virus-host cell interaction.

A second type of change in the cell genome can be quantitatively scored by the single-cell survival technique; namely, the expression of a virus resistant state. Resistance to virus has been accurately assessed in this manner by Puck and Cieciura (33) with the NDV carrier state in HeLa; and by Vogt (44), Vogt and Dulbecco (45), Darnell (7), and Darnell and Sawyer (8) for poliovirus-resistant HeLa lines.

It is noteworthy that strains of HeLa resistant to NDV, excepting the virus carrier type (5, 6, 33), have not been found even though, to date, cell numbers in excess of 10^9 have been screened. In marked contrast, investigators working with the poliovirus-HeLa system have experienced relatively little difficulty in securing lines of HeLa resistant to that virus. Clonal lines of HeLa have been isolated whose survival curves reflect cell killing by only 1 out of 10 to 20 of the poliovirus particles scored on the parental cell population (7, 44, 45). In this connection, as we have previously pointed out (23), the terms "sensitive" and "resistant" alone, are not as useful when applied to virus-host cell relationships in animal virus systems as in bacteriophage, where the response is usually all-or-none. Care should be taken in animal virus studies always to specify precisely the test situation under which "sensitivity" or "resistance" occurs.

B. The Virus Genome

As with the cell genome, changes in the genetic constitution of the virus may have noticeable effects on the cell-killing reaction. Most striking in this respect are the results obtained with a strain of NDV (Mass.-HiK) that inherently produces particles which display a low order of elution activity. For example, at 37 C, pH 7.4,

and a plaque-forming particle to chicken red blood cell ratio of 0.04, where more than 98 per cent of the cells which attach virus have only 1 particle, maximal elution of particles of the Beaudette strain is achieved within less than 10 min. The "slowly eluting" Mass.-HiK strain does not attain the maximal level of elution until hours later, and then only at about 1/5 the value of the Beaudette strain. Elution of NDV-(Mass.-HiK) from host cells such as HeLa is essentially lacking, even under conditions which produce significant elution of NDV-Beaudette particles (see below and table 2). The Mass.-HiK/ strain of NDV was kindly supplied by Miss Leila Diamond and Dr. Alice Moore, and was originally isolated by these workers through selection on ascites tumor cells (28). The availability of this strain provides an unusual investigative instrument. For example, we may now examine host cell interaction with a Newcastle disease virus member of the myxovirus family comparatively free of the reactions attributable to virus elution, and thus better define the role of elution (elution enzyme activity) (*cf.* 16). The following experiments relate to the function of elution enzyme: when low multiplicity cell survival curves for the "slow-eluting" strain were determined, first with the dual temperature sequence (attachment at 0 C and penetration at 37 C), and secondly entirely at 20 C, a rather surprising result was obtained. The "slowly eluting" strain produced a 3.5-fold reduction in slope of survival curves run at the lower temperature, as seen in Figure 6. Thus, out of an average of 3.5 killing particles per cell expressing successful penetration at 37 C, on the average, only 1 completes entry into the cell at 20 C. Three strains of NDV containing high enzyme activity, measured by their rates of elution from chicken red blood cells as described above, showed at most a 30 per cent reduction in the slope of survival curves.

As a working model upon which to base future experiments, these results are interpreted as follows. The concentration of elution enzyme in NDV particles containing this activity at the usual level appears to be of such magnitude as not to limit the penetration rate to a significant extent at the lower reaction temperature. (Enzyme activity is such that even at 0 C when near-saturating doses of virus are added to chicken red blood cells, complete destruction of

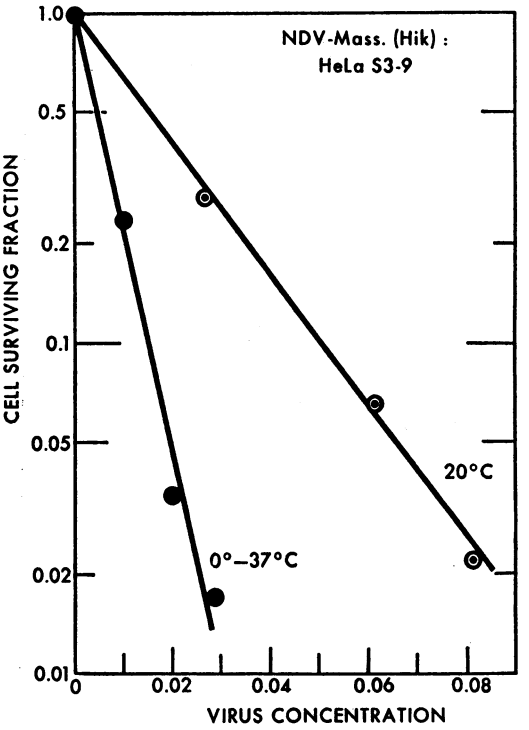


Figure 6. Survival curves of HeLa S3-9 cells exposed to a slow eluting strain of Newcastle disease virus (NDV) at different reaction temperatures. NDV-strain Mass. (HiK) was allowed to react with S3-9 cells under two conditions: (a) (*upper curve*) Virus-cell complexes were formed and held at 20 C prior to plating for survivors, and (b) (*lower curve*) Virus-cell complexes were formed at 0 C, and diluted and maintained at 37 C before plating for survivors.

receptors occurs within minutes (Marcus and Tolmach, *unpublished data*)). Also, these results suggest that some rate-limiting process essential to the penetration reaction, and dependent upon enzyme activity for completion, is controlling virus entry at the lower temperature. Irrespective of the details of the mechanism operating to complete virus particle entry into the cell, we might expect a marked reduction in the rate of this process with a 17 C drop in temperature, such that with time, in the absence of significant enzyme activity, firmer virus-to-receptor binding may ensue of the type suggested by Burnet and Lind (3). Thus, these firmly bound particles would still be situated on or near the cell surface and succumb to the virucidal action of the growth medium at the time of plating.

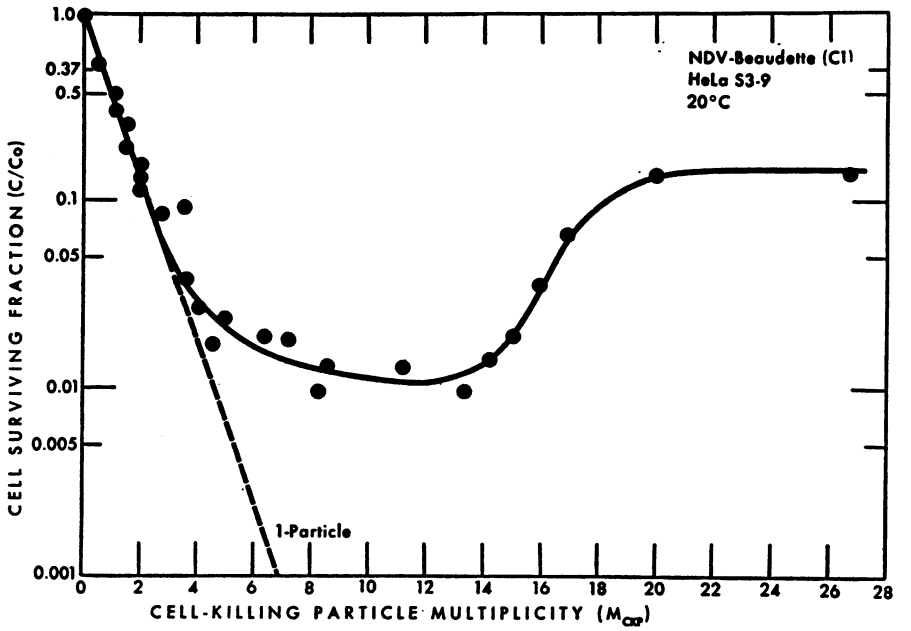


Figure 7. Survival curve of HeLa S3-9 cells exposed to Newcastle disease virus (NDV), strain Beaudette at 20 C; the cell-sparing phenomenon. NDV-Beaudette at various concentrations was attached to S3-9 cells (1.0×10^7 per ml) for 30 min at 20 C prior to dilution and plating as usual. The theoretical 1-particle-to-kill curve is included for comparison.

Preliminary inactivation rate studies with the fast and slowly eluting strains of virus indicate that the latter may be more sensitive to the virucidal constituents of the plating medium, suggesting a possible inhibitor-destroying function of the enzyme.

It is interesting to note that the survival curve determinations at 20 C with the slow eluting strain of NDV mimic the response of resistant cell lines of the type described by Vogt (44), Vogt and Dulbecco (45), and Darnell (7) for poliovirus-HeLa cell interaction carried out entirely at 37 C.

C. Virus Multiplicity and Reaction Temperature

1. Cell-sparing phenomenon. When virus-cell complexes of the Beaudette strain of NDV are formed at various cell-killing particle multiplicities at *20 C* and plated into virucidal growth medium in the standard fashion, results of the type shown in figure 7 are obtained. Up to an average of 3 to 4 ckp per cell, the fraction of survivors follows the theoretical 1-particle-to-kill relationship. Adsorption of greater than this number of particles results in a surviving cell fraction in excess of that expected by any one-hit process. Surprisingly enough, if the average number of ckp attached per S3-9 cell exceeds about 14, then the number of survivors actually increases, indicating that the probability of a cell's being killed when it initially attaches, on the average, 14 or more killing particles is less than when a smaller number is bound, *e.g.* from 5 to 13. This type of reaction is reminiscent of the reports of ameliorated host response to large doses of virus (4, 14, 18).

We investigated what parameters might influence the magnitude of this cell-sparing effect. The following experiment was performed. NDV (Beaudette)-HeLa complexes at a ckp multiplicity of 19 were formed at 0 C, instead of the usual 20 C. At the end of a 30-min attachment period, the 0 C virus-cell complexes, still at this low temperature, were washed free of unadsorbed virus, and samples were removed, diluted, and set at various temperatures for an additional 30 min before plating to determine surviving cells. Figure 8 shows the results of a typical experiment. Here it is seen that the maximal fraction of cells spared from the lethal action of

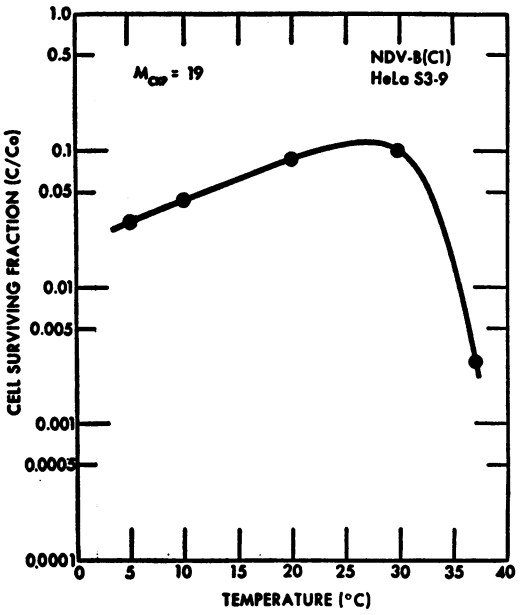


Figure 8. Survival of HeLa S3-9 cells exposed to Newcastle disease virus (NDV)-Beaudette at different temperatures of penetration. Virus was attached to host cells at 0 C such that $m_{ckp} = 19$. Following several washings, samples of the complexes formed at low temperature were diluted into medium at various temperatures, held for 30 min, and plated as usual for survivors.

virus occurs between 20 and 30 C. Quite strikingly, the maximal expression of cell killing obtains when the virus-cell complexes formed at 0 C are held at 37 C before plating. Although the theoretical kill for a ckp multiplicity of 19 was still not realized, the value obtained showed a 30-fold increase in cell killing and suggested the conditions to obtain maximal expression of virus action. Consequently, cell survival curves were run where virus-cell complexes were formed at 0 C and then rapidly diluted into a nonvirucidal medium at 37 C to complete the penetration reactions prior to plating in the virucidal growth medium. Dilution of the virus-cell complexes also serves to circumvent the cell agglutination which occurs at 37 C (23). Figure 9 illustrates such a survival curve for high multiplicities of NDV-B on HeLa S3-9. It is clear that the cell-sparing effect, *i.e.*, the unexpected rise in cell survival as virus multiplicities are raised, has been eliminated. However, there is still anomalous survival in excess of the theoretical when an average of 3 or more particles are attached per cell.

Previously presented evidence suggested that failure of the virus penetration reaction may play a role in the cell-sparing phenomenon (24). In this case, the presence of elution enzyme on the virus was proposed as contributing to abortive penetration processes. Experiments with chicken red blood cells and elution enzyme-containing virus make clear that the rate of virus elution is greatly enhanced by high multiplicities (13, 41). The recent work of Sagik and Levine (40) with the Beaudette strain of NDV and chicken red blood cells further substantiates this relationship. Therefore, it is not unlikely that the high ckp multiplicity required to produce the cell-sparing effect is so related. To test this premise, NDV-B was attached to HeLa cells at 0 C in both low and high multiplicities and the resulting virus-cell complexes were washed 5 times, set at 20 C, and tested after 30 and 60 min for eluted virus as plaque-forming particles. The results are shown in table 2, where it is seen that at both low and high multiplicities the fraction of virus attached was the same; however, after 30 min at 20 C, only 0.10 per cent of the total bound virus was released from the low multiplicity virus-cell complexes, whereas at the higher input, 12.2 per cent was measured as free virus, *i.e.*, over 100 times as much virus as at the low multiplicity. No further elution occurred with continued incubation. Tests after 60 min at 20 C show that both low and high multiplicity complexes were able to accept a second input of virus to essentially the same extent as they had originally. This latter finding suggests that only a small fraction of the cells contribute free virus through receptor-site destruction, and that in terms of attachment kinetics, their contribution to a reduction in the rate of virus uptake would be insignificant. If the total yield of eluted virus from the high multiplicity complex mixture is assumed to come only from those cells which are spared, then the average number of virus particles released per cell is about 18, a value consistent with a model in which the probability of escaping death is greatest in those cells which simultaneously bind a large enough number of enzyme-containing particles to initiate total elution of virus and thus abort penetration by even one lethal particle.

It is noteworthy that the fraction of bound NDV-B which elutes from HeLa at the higher multiplicity is in close agreement with that reported to elute from chick embryo cells which

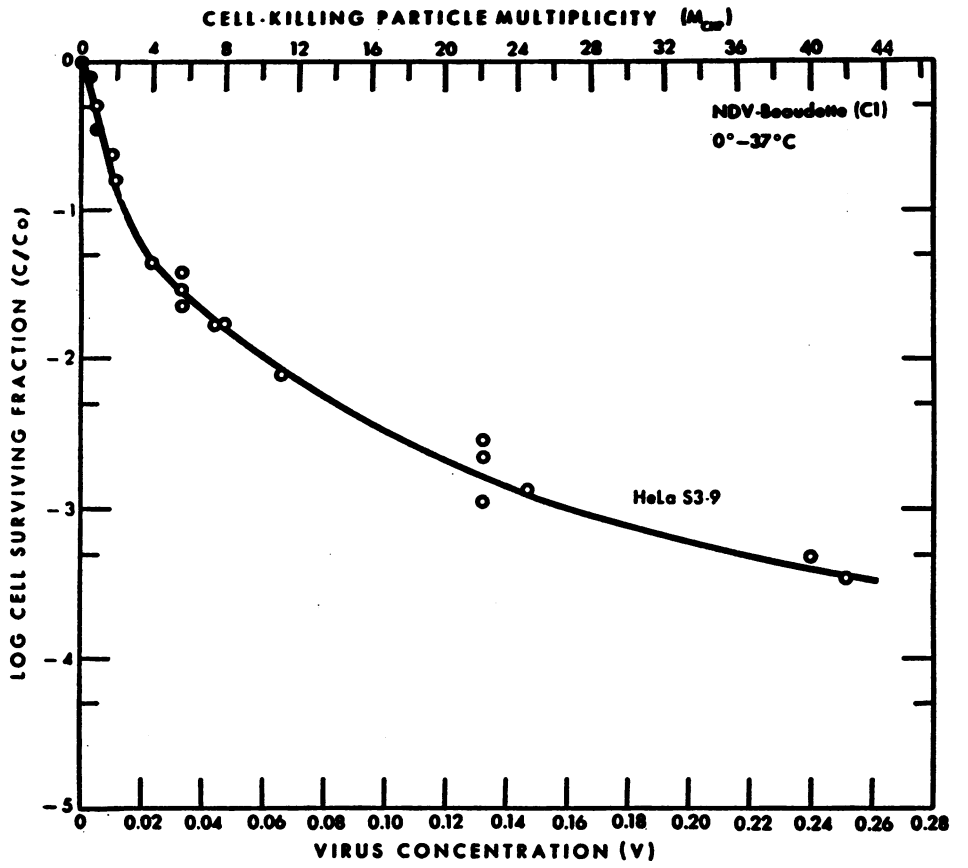


Figure 9. Survival curve of HeLa S3-9 cells exposed to high multiplicities of Newcastle disease virus (NDV)-Beaudette with the dual temperature sequence (0 to 37 C). Virus-cell complexes were formed at 0 C for different concentrations of virus, rapidly diluted into medium at 37 C, and held for 15 min prior to plating for survivors.

TABLE 2

Newcastle disease virus-Beaudette elution from low and high multiplicity virus-HeLa cell complexes

Multiplicity (First Input)	Virus Attachment (First Input)	Eluted NDV: of Total Attached Virus		Virus Attachment (Second Input: After 60 Min at 20 C)
		30 Min	60 Min	
pfp*	%	%	%	%
2.2	64.0	0.10	0.15	53.2
16.3	62.6	12.2	10.1	55.0

* pfp = Plaque-forming particle. Attachment at O C. Elution at 20 C.

have adsorbed a similar number of particles (20). It has been demonstrated that in general, spontaneous elution of myxovirus particles bound in low numbers to host cells, as opposed to red blood cells, does not occur readily (20). Our own experiments (*cf.* table 2) support these findings.

If indeed elution or more precisely the presence of eluting enzyme on the virus particle does contribute to the cell-sparing phenomenon, then a strain of NDV with reduced elution activity, like that described above, would not be expected to display this effect. A survival curve of this variant was run at 20 C to maximize the cell-sparing reaction and the results shown in figure 10 were obtained. The most striking aspect of the curve is the absence of any reversal in the decline of the surviving cell fraction as the virus multiplicity is raised. In fact, this 20 C survival curve with the slow eluting virus resembles in shape that obtained with the fast eluting strain, Beaudette, when the dual temperature procedure was employed (*cf.* figure 9). Two other strains of NDV with substantial enzyme activity, in-

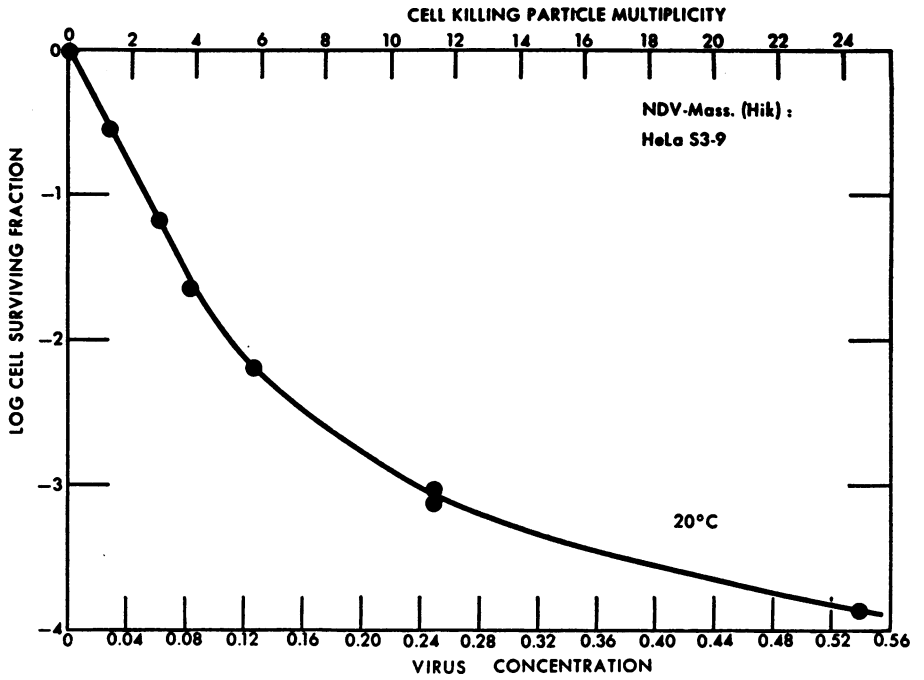


Figure 10. Survival curve of HeLa S3-9 cells exposed to high multiplicities of Newcastle disease virus (NDV)-Mass. (HiK) at 20 C. Attachment and plating conditions were as described for figure 7 except that the slow eluting strain of NDV was used.

cluding the parent genotype of the slow eluting strain, showed increased cell sparing at high multiplicities when tested at 20 C.

The phenomenon of cell sparing appears to be analogous to "recovery" of Ehrlich ascites tumor cells from the lethal action of NDV, as described by Moore and Diamond (27), Moore *et al.* (29), and Prince and Ginsberg (32). The following considerations, based largely on results from cell-plating experiments, agree in many respects with conclusions reached by these workers concerning the "recovery" effect.

2. On the mechanism of cell sparing. From the above considerations it seems that at least a large part of the deviation from a one-hit kill, which is observed at *high multiplicities* in experiments carried out at *20 C*, is attributable to the presence of *elution activity* of the virus. This suggests that the simultaneous attachment of a large number of elution enzyme-containing particles, probably in excess of 14, can prevent successful penetration of the cell by even 1 killing particle. Virus elution, leading to cell sparing, and virus penetration terminating in cell death, probably compete with each other. The penetration reaction appears to be maximally efficient even when only 1 particle is attached per cell, since the good agreement with theory in survival curve analyses at low multiplicities shows that the probability of successful penetration and, therefore, lack of elution, is essentially one when only a few particles are present per cell. With increasing virus multiplicity, contributions by the various enzymatic activities become additive and mutually frustrating to the penetration process. At 20 C the combined enzymatic activity of a large number of particles will suffice to prevent penetration by any of the particles and thus result in cell sparing. If this is accompanied by receptor-site destruction, the cell should become refractory to superinfection until new receptor sites make virus attachment again possible. The experiments in the following section relate to this point, and also appear to explain a similar phenomenon described by Prince and Ginsberg for the NDV-Ehrlich ascites tumor cell system (31), detailed discussion of which is forthcoming.

D. Recovery Experiment

1. Recovery in active virus-treated cells. Over 25 colonies which had developed from cells receiving an average of 20 ckp in a 20 C experiment were tested for their response to infection with low

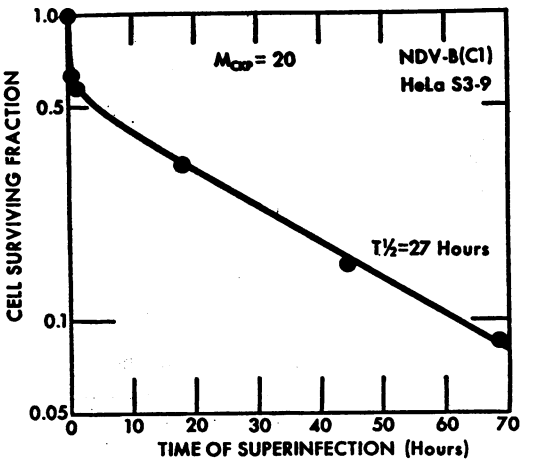


Figure 11. Survival of spared-HeLa S3-9 cells exposed to a challenge dose of Newcastle disease virus (NDV) at various time intervals. S3-9 cells which initially had attached 20 cell-killing particles at 20 C were plated as usual in a complete growth medium at 37 C, but at various intervals thereafter a challenge dose of virus large enough to kill all untreated cells was added and the fraction of survivors was determined in the standard manner.

virus multiplicities. All of these colonies proved sensitive. The resistant state resulting in cell sparing was therefore not genetic, but reflected a transient physiologically refractory condition, which was lost upon regrowth. Sometime between the initial attachment of the virus and retesting of the surviving colonies 8 to 9 days (10 generations) later, the cells had regained their original sensitivity to the lethal action of the virus. The following experimental procedure was designed to study the kinetics of the change from the resistant to the sensitive state. A suspension of HeLa cells was exposed to a high ckp multiplicity and the resulting virus-cell complexes were plated in the standard fashion with each plate receiving about 200 physiologically resistant cells. At various intervals thereafter, with the virus-cell complexes at 37 C, a second dose of virus was added in the lowest concentration that would just produce complete killing of untreated cells. The virucidal action of the growth medium was such that within less than 10 to 30 min no residual challenge virus activity remained. These recovery experiments permit an accurate determination of the average rate of cell reversion from the refractory to the sensitive state. Figure 11 shows the results from one such experiment. About one half of the population is immediately susceptible to superinfection, suggesting that at the very early challenge times the refractory state has not yet been reached in this fraction of cells. The remaining cells return to a sensitive state with a half-time of about 27 hr, a period comparable to the generation time (20 hr) of uninfected cells in the given medium.

2. Recovery in neuraminidase-treated cells. Experiments similar to those described above were carried out with neuraminidase (receptor destroying enzyme, RDE)-treated cells, in order to determine whether receptor-site destruction by this enzyme would yield cells behaving like the refractory cells produced from action of the virus. Here, HeLa cells were exposed to neuraminidase (10) (generously supplied by Dr. F. M. Burnet) for 30 min at 37 C, plated in the standard fashion at 200 cells per plate, and challenged

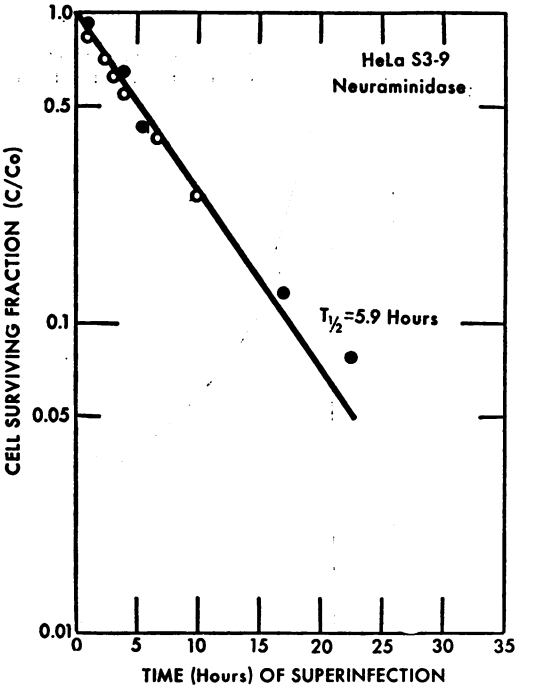


Figure 12. Survival of neuraminidase-treated HeLa S3-9 cells exposed to Newcastle disease virus (NDV) as a function of time after treatment. A monodisperse suspension of 2.0×10^6 S3-9 cells per ml was treated with approximately 6000 units of neuraminidase per ml for 30 min at 37 C, seeded in a complete growth medium at 200 cells per plate, and at various intervals thereafter active NDV was added in a concentration sufficient to kill all untreated cells.

at intervals by adding the same dose of virus previously employed. Addition of the challenge virus dose immediately after termination of enzyme treatment showed that as high as 85 per cent of the cells were immediately refractory to the lethal action of NDV. Unchallenged control cells plated with 100 per cent efficiency. The rate at which these cells return to a susceptible state, presumably a measure of receptor-site repair or synthesis, is shown in figure 12. The average half-time of recovery in two experiments was 5.9 hr.

3. On the mechanism of recovery. The wide differences in the relative rates of reversion from the refractory to the sensitive state shown by the two different inducing agents seem to be understandable in light of the following considerations. The relative lability of neuraminidase and its demonstrably vanishing activity upon dilution into growth medium suggest a rapidly destroyed enzyme activity once the cells have been plated. Thus, the short half-time of return to sensitivity displayed by neuraminidase-treated cells seems to reflect the maximal average rate at which receptors may be resynthesized, or replaced, in the absence of competing enzyme action. The much slower return to sensitivity of virus-treated cells suggests that receptor-site destruction by virus-situated enzyme may represent a more prolonged action, *i.e.*, include more extensive receptor-site disorganization, or produce more deep-seated changes in the cell membrane than the free enzyme, and so require a longer repair time. It is conceivable also that the refractory cells represent members of the host population with physiologically altered characteristics concerning the number or distribution of receptor sites, or other cell surface elements which

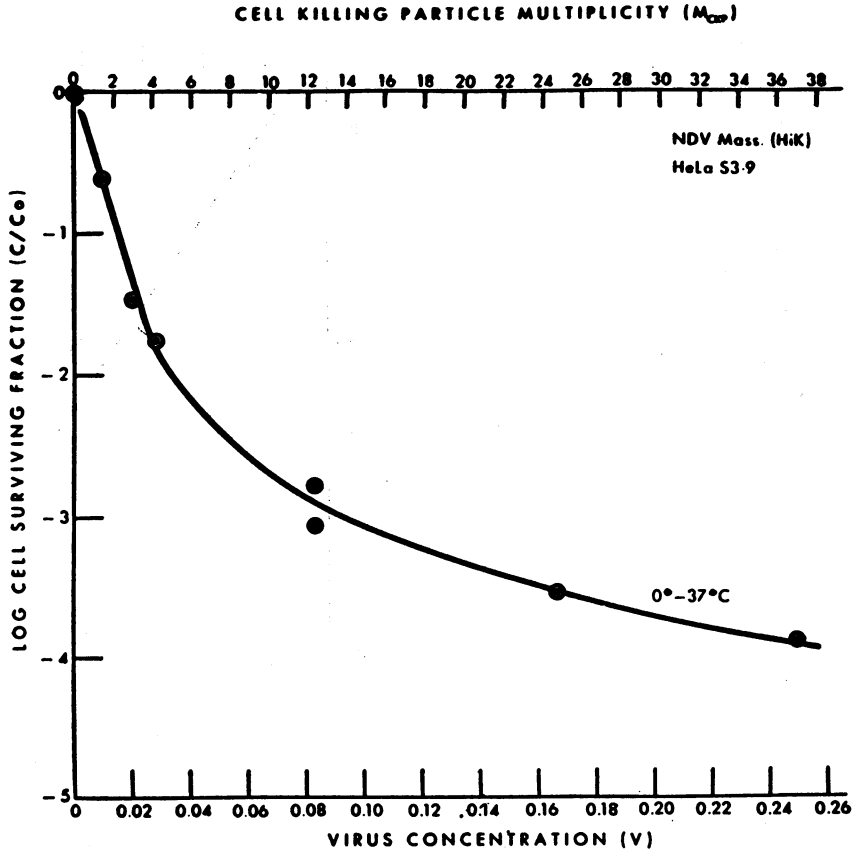


Figure 13. Survival curve of HeLa S3-9 cells exposed to high multiplicities of the slow eluting strain of Newcastle disease virus (NDV) with the dual temperature sequence (0 to 37 C). Virus attachment, penetration, and cell plating conditions as in figure 9.

may be differentially involved in the reactions of penetration and elution.

E. Anomalous Survival at High Multiplicity with the Dual Temperature Sequence

Although a competition between the rates of virus elution and penetration may account for cell sparing when virus and cell interact in high multiplicity at 20 C, it leaves unexplained the mechanism underlying the apparent resistance of cells to high virus concentrations as displayed in the 0 to 37 C sequence experiments. Here, both fast eluting and slow eluting strains of NDV produce essentially the same shape of survival curve (*cf.* figures 9 and 13), thus relegating to a minor role the elution reaction. To date, all colonies developing from cells exposed to ckp multiplicities as high as 50 with the dual temperature sequence have proved to be only transiently resistant and, like survivors from the cell-sparing reaction, display complete sensitivity upon regrowth.

F. Induction of a Refractory Cell State by Ultraviolet-Irradiated Virus

Finally, we examined the development of a refractory state induced in HeLa through the adsorption of ultraviolet-irradiated NDV (UV-NDV). In our earlier work (24) we reported that UV-NDV did not kill cells, although such particles were fully functional with respect to attachment and enzyme activity. These attributes combine to give us a nonlethal carrier of virus elution enzyme, whose action on the cell we can compare with free enzyme (neuraminidase) or with active virus enzyme.

When UV-NDV is attached to HeLa at a multiplicity of 3, in the cold, and the temperature of the complex raised to 37 C, the number of cells attaining a refractory state doubles every 9 min as shown by application of a virus challenge at various intervals to a series of tubes containing monodisperse complexes. These results are illustrated in figure 14.

1. Recovery in UV-NDV-treated cells. Once the refractory state was produced by UV-NDV, the rate at which induced cells returned to susceptibility was determined by a recovery experiment, as in the case of free enzyme or active virus. The susceptible state was found to return very slowly, with a half-time of about 100 hr. A recovery curve for UV-NDV-treated cells is presented in figure 15, in comparison with those for free enzyme and active virus.

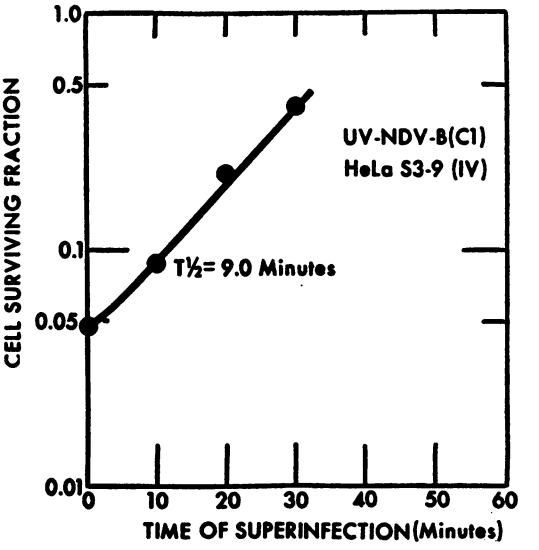


Figure 14. Time course of development of a refractory state in HeLa S3-9(IV) cells exposed to ultraviolet-irradiated Newcastle disease virus (NDV). Cell-killing particles of NDV-B which had received 4-lethal hits (e^{-1} doses) of ultraviolet irradiation were attached to HeLa cells at a multiplicity of 3, and the resulting virus-cell complexes incubated at 37 C for various intervals. Development of a refractory state to superinfection was measured by periodically adding a challenge dose of active NDV large enough to kill 95 per cent of the unincubated complexes, and scoring the surviving fraction of cells in the usual manner.

2. On the mechanism of a UV-NDV-induced-refractory cell state. When populations of UV-NDV-treated HeLa cells were tested for their ability to attach a second input of virus, it was found that the fraction of virus adsorbed diminished with time as the UV-NDV-cell complexes were held at 37 C. This relationship is illustrated in figure 16. Thus, it seems that destruction of receptors, as in the case of free enzyme treatment, explains the UV-NDV-induced refractory state, a condition similar to that recently described by Baluda (2) for chick embryo cells in monolayer culture.

The slow rate of conversion from refractory to sensitive state for UV-virus-treated cells is consistent with prolonged virus-enzyme-cell contact. Its extension over several cell generations

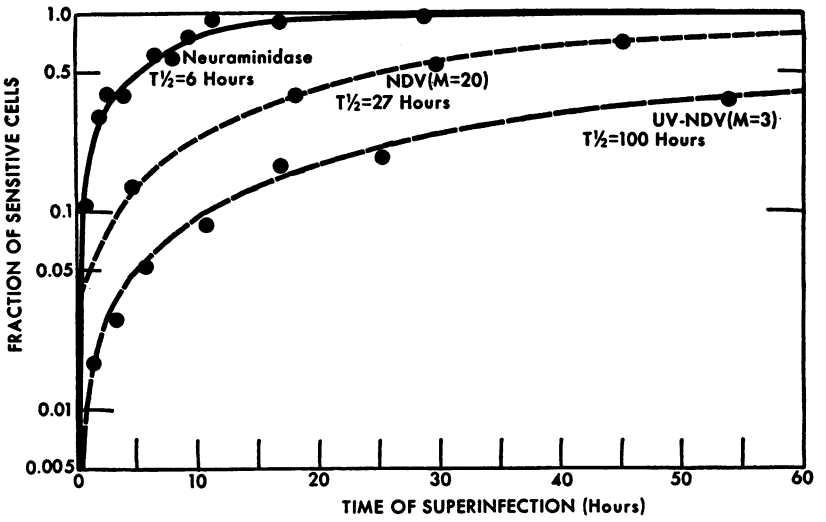


Figure 15. Summary of the kinetics of recovery from a virus-refractory to a virus-sensitive state of neuraminidase-, active Newcastle disease virus (NDV)-, and ultraviolet (UV)-NDV-treated HeLa cells. Recovery from the refractory state was measured as described in figures 11 and 12. The fraction of sensitive cells was calculated from $1 - C/C_0$ and plotted as a function of time of superinfection.

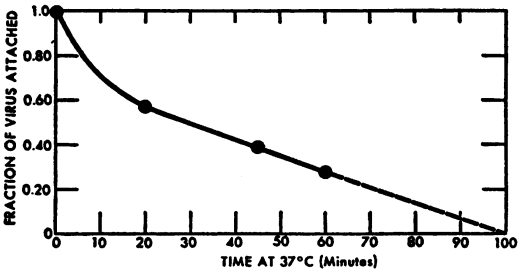


Figure 16. Rate of loss of virus-binding capacity of HeLa cells exposed to ultraviolet-irradiated Newcastle disease virus. HeLa cells which had attached 3 cell-killing particles inactivated by 4-lethal hits (e^{-1}) of ultraviolet light were incubated for various periods at 37 C (abscissa). The ability of cells so treated to adsorb a second input of (active) virus was measured by plaque-forming particle disappearance from the supernatant fluid of the virus-cell mixture after a constant period of attachment, *e.g.*, 30 min. Cell density = 1.0×10^7 per ml.

suggests the persistence of enzyme activity even after cell division and seems compatible with the continued presence of enzyme, or virus-contained enzyme on or near the cell surface. This concept is supported by our demonstration that (a) only a very small fraction of virus attached at low multiplicity can be eluted, (b) there exists a high efficiency of virus penetration at low multiplicities, and (c) active attached virus rapidly becomes inaccessible to inactivation by growth medium, a condition which invites continuous enzyme activity from a center(s) so situated cellularly as to resist inactivation by serum.

A possible physical picture for establishment of a protracted refractory state might involve entry of UV-damaged ribonucleic acid of irradiated NDV into the cell, where it initiates a self-limiting production of new virus devoid of killing power, but competent in enzyme content (*cf.* Henle *et al.* (15)), whose continued presence during the release cycle of new virus suffices to maintain surfaces free of receptors. Particulate units of enzyme (hemagglutinin) of the type described by Granoff (11) and Granoff and Henle (12) as possible virus particle precursors might provide the means of distributing and maintaining a sufficient level of enzyme activity over several doubling dilutions, *i.e.*, cell divisions. Their demonstrated tenacious association with cells of the allantoic membrane also favors persistence through cell division. Enzyme packages of the Granoff-Henle type of particle may even be present at a very early stage, originating from the initial infecting particle(s).

In connection with the refractory state, Schlesinger (42) suggests that the maintenance of refractoriness to superinfection in inter-

ference reactions depends on the *continued association of the interfering virus (agent) with the host cell.* Our experiments seem to support this concept.

The picture of virus-cell-enzyme interaction we have just proposed as a working model seems to account for the main characteristics of the NDV-carrying HeLa strain. As demonstrated by Cieciura (5) and Puck and Cieciura (33), it is refractory to superinfection primarily because of an apparent lack of receptors, yet active virus is always demonstrable, intimately associated with the cell. Still unanswered is the exact nature of the physiologic and/or genetic relationship between potentially lethal virus and susceptible host which tolerates a temperate-like existence.

Finally, the induction of a refractory state in host cells by low multiplicities of UV-NDV appears to differ from the classical example of receptor site destruction and subsequent virus elution in the erythrocyte systems, in that the virus particle remains firmly bound to the host cell and apparently may initiate a continuous receptor-destroying action.

SUMMARY

The establishment of routine quantitative animal cell plating techniques in which monodisperse cells behave as do bacteria in standard bacteriological procedures, affords additional criteria to score virus-host cell interactions. One such criterion, based on the colony-forming response of single HeLa cells exposed to various concentrations of virus, provides the experimental approach needed to determine the kinetics of cell killing by animal viruses and is described herein. The one-hit nature of the survival curves obtained by the single-cell survival procedure demonstrates the lethal capacity of a single virus particle for an individual host cell, and in this respect, duplicates exactly the classic experiments of Luria and Delbrück with bacteriophage (21). As in their system, it was shown that cells which receive a lethal dose of virus do not undergo even one reproductive division.

Pronounced changes in the virus genome, as reflected by a marked reduction in elution capacity, were revealed in various ways through the kinetics of cell killing. These suggested a possible role for elution enzyme. More subtle differences in genetic constitution of a reacting member, the host cell, were detectable through survival curve analyses of different host strains, which point out that, in addition to detecting killing particles, the single-cell survival procedure permits exact definition of host cell response in terms of "resistant" or "sensitive" states. It also emphasizes the basic differences between the usual response of bacterial and animal cell hosts to invading viruses.

Recovery-type experiments afford means of determining the kinetics of cell reversion from temporary physiological states of resistance to those of sensitivity and provide an accurate measure of the average rate of receptor-site repair (resynthesis) after various receptor-destroying treatments. The recovery experiments with free enzyme- and virus-treated cells have uncovered a marked quantitative difference in reversion rates which, coupled with data on cell killing and virus penetration, point out the tenacious binding of virus to cell in low multiplicity infection and provide a model of virus-host cell interaction during sustained contact and receptor destruction through persistent enzymatic activity. The NDV-carrying HeLa cell is discussed in light of this model.

Cell-killing experiments also revealed an unexpected phenomenon—cell sparing—under conditions which favor virus elution, *e.g.*, high virus to cell ratios, reaction temperatures around 20 C, and high elution enzyme activity.

Although maximal agreement with the 1-particle-to-kill relationship was found to obtain when virus was attached in the cold and allowed to penetrate at 37 C, at certain high virus multiplicities, determined by the genetic constitution of the host cell, anomalous survival in excess of that theoretically expected was observed. This effect was not accounted for by the mechanism used to explain the cell-sparing reaction.

Comparative assays of plaque-forming and cell-killing particles for different virus strains indicate that detection of the latter may be possible under conditions which do not score all infectious entities. The cell-killing property is shown to be extremely stable to low temperature storage but sensitive to heat and ultraviolet radiation. Finally, the unique attribute of scoring killing particles, namely, that a virus particle need only prevent cell reproduction, and not carry out successive waves of infection, may make possible detection of more elusive virus forms.

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