Mechanism of Interferon Induction by NDV: a Monolayer and Single Cell Study

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SUMMARY

Newcastle disease virus (NDV) stocks contain three types of particles which were tested for interferon inducing ability: (1) plaque-forming haemagglutinating particles, (2) non-plaque-forming haemagglutinating particles containing RNA and (3) non-plaque-forming haemagglutinating particles which contain no RNA. Single NDV-infected cells were isolated in microdrops and tested for interferon production as measured by protection of 30 ± 6 additional cells from challenge with mengovirus. This technique demonstrated that: (1) essentially every cell can be induced by plaque-forming NDV to produce interferon, (2) neither of the two types of non-plaque-forming haemagglutinating particles were capable of inducing detectable levels of interferon and (3) while infection with a single active virus was sufficient to induce interferon, the efficiency of induction increased with increasing multiplicity. It was concluded that some virus synthetic processes are probably required to initiate induction.

INTRODUCTION

Non-replicating virus can induce interferon. Evidence for this dates from the original demonstration of interferon production in cells infected with heat-inactivated influenza virus by Isaacs & Lindemann (1957), and includes work with heat-inactivated arboviruses (Chikungunya virus, Gifford & Heller, 1963), Sindbis virus and Western equine encephalomyelitis viruses (LeClerc & Cogniaux-LeClerc, 1965); u.v.-inactivated viruses including NDV, influenza, fowl plague, mumps and Sendai viruses (Henle et al. 1959; Youngner et al. 1966; Gandhi & Burke, 1970) and hydroxylamine-inactivated Sendai virus (Goorha & Gifford, 1970).

Furthermore, Rodriguez & Henle (1964) who studied the induction of interferon in L cells by the Victoria strain of NDV, concluded that the naturally occurring non-infectious (i.e. non-plaque-forming) virus in NDV stocks was the sole inducer of interferon in this system. Similarly, Gifford (1963) presented evidence that interferon induction by Chikungunya virus was due to inactive viruses in the virus preparation and, later, Toy & Gifford (1967) made similar claims for Semliki Forest virus. In both these latter cases, optimum interferon induction occurred at input multiplicities of less than 1 p.f.u./cell. In a later study, Goorha & Gifford (1970) infected chick embryo cells with either 10 or 0.1 p.f.u./cell of Semliki Forest virus and found that the interferon yields were equal at 8 h post-infection.

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time when only one cycle of replication could have occurred. These studies, however, were
open to two possible interpretations: naturally occurring non-plaque-forming particles
induced interferon, or multiple infection of a cell with plaque-forming virus reduced the
interferon yield to one tenth the amount expected for a singly infected cell. Thus, while it is
clear that artificially inactivated virus particles can induce interferon it is not certain whether
the non-plaque-forming particles found in an untreated lysate can do so.

Whatever type of particles induce interferon, virtually all of the infected cells are destroyed,
even if large amounts of interferon are produced. However, a few survive (Marcus, 1959)
possibly because they are in a favourable metabolic state (for example mitotic metaphase
(Marcus & Robbins, 1963)), and since interferon is effective in very low quantities (Paucker
& Stancek, 1972) it is possible that the few surviving cells produce all of the interferon found
in the culture. Indeed, it has been shown that a few interferon producing cells can protect a
whole culture (Gresser & Enders, 1962). Hence, a proper understanding of the induction
process requires a knowledge of the proportion of cells in an infected culture that are actually
producing interferon.

The NVD-L cell system is uniquely favourable for investigating these questions. NDV
induced high titres of interferon in L cells, and its stocks contain several types of particles
which can be tested separately for interferon inducing ability. Furthermore, it is possible to
isolate individual L cells in microdrops after infection with NDV, and by assaying them for
the presence or absence of interferon, determine the proportion of cells which are producing it.

METHODS

Cells and viruses. L cell strain 929 was obtained from the Dow Chemical and Pharma-
ceutical Company (Zionsville, Indiana). For plaque assays, interferon assays, and micro-
drop experiments, cells were seeded on 60 mm plastic Petri plates (Falcon Plastics).

Chick embryo cell (CE) plates were prepared from 10-day-old embryos as previously
described by Franklin, Rubin & Davis (1957).

Chick red blood cells (RBC) were collected from adult white leghorn roosters via a heart
puncture into Alsever’s solution, washed and stored at 4 °C in phosphate buffer.

Mengovirus, originally obtained from Dr Richard Franklin, was selected for heat resist-
ance (8 h half-life at 37 °C), plaque-purified, and grown in L cell monolayers. After 18 to
24 h, the monolayers were disrupted by freeze-thawing three times, and the supernatant
medium was stored at −60 °C. Virus titres were generally about $1 \times 10^9$ p.f.u./ml.

Light-sensitive mengovirus was prepared by infecting the cells in the presence of 4 µg/ml
neutral red (Miner, 1965). After a second growth cycle in the presence of neutral red,
the light-sensitive virus could be inactivated by a factor of $10^7$ by exposure to a 15 W
fluorescent white light source at a distance of 10 cm for 8 min. Virus titres were generally
1 to $3 \times 10^6$ p.f.u./ml.

NDV (Beaudette strain) stocks were prepared in 10-day-old chick embryos by injecting
$10^4$ p.f.u. into the allantoic sac. The allantoic fluid was harvested 36 to 48 h post-infection.

Assay systems. Mengovirus was assayed on day-old L cell monolayers. After 40 to 60 min
adsorption at 37 °C, 5 ml of a starch overlay (DeMaeyer & Schonne, 1964) was added, and
the cells were incubated at 37 °C for 24 to 28 h. NDV plaque assays were performed as
above, except that CE cells were employed, an agar overlay was used, and incubation was for
48 h. In both cases, cells were stained by adding 1 ml of an 0.2% neutral red solution in
phosphate buffer to each plate. Plaques were counted after 2 h (see Fleischmann & Simon,
1973 for details).
Interferon induction

Media. L cells were grown in Eagle's minimal essential medium (MEM, Schwartz-Mann) supplemented with 10% newborn calf serum (Grand Island Biological). For CE monolayers, the above medium was supplemented with 5% of a tryptose phosphate broth (29.5 g/l). A 'super-conditioned' medium prepared from the supernatant fluid of u.v.-irradiated L cells as previously described (Fleischmann & Simon, 1973) was used for all microdrop work.

Interferon preparation and titration. L cell monolayers were infected with NDV, and incubated for 24 h. The supernatant fluid was collected, and residual virus was inactivated by dialysis at pH 2 for 12 h at 4 °C in a 0.05 M-KCl solution. Following further dialysis against a phosphate buffer at pH 7.2, the material was stored at 4 °C. In our hands the NDV titre observed before dialysis was 10^5 p.f.u./ml and treatment at pH 2 reduced live NDV in the interferon preparation to less than 10 p.f.u./ml. The crude preparation was not purified, but was generally used at dilutions of 100 or more, which minimized the influence of impurities. Reconstruction experiments in which different amounts of NDV were added to culture medium which was then assayed for 'interferon' activity showed that at least 10 p.f.u./ml of NDV were required to inhibit plaque formation. Hence, even if residual virus activity was present, it would not have affected our results.

Interferon titres were determined by a plaque reduction assay (Wagner, 1961) and expressed as the reciprocal of that dilution, in 2 ml, which reduced the number of mengo-virus plaques to 50% of the control. Titres of 1000 to 2000 units were routinely obtained. Under the same conditions, ten units of two separate lots of reference research standard mouse interferon titred as 1 ± 0.2 units. Our assay for interferon is apparently only one tenth as sensitive, as that used for the research standard.

Sucrose gradient techniques. The general techniques are given in Dahlberg & Simon (1969). 30 to 50% sucrose gradients were spun at 16000 rev/min for 2 1/2 h and 15 to 30% gradients were spun at 20000 rev/min for 40 min. Trailing virus from the main infectivity peak was avoided by harvesting the 15 to 30% gradients from the top of the tube by means of an ISCO gradient analyser.

Haemagglutination assay for NDV. The methods are described by Isaacs (1957), who determined that the ratio of physical particles to RBCs at the endpoint is approx. 1:1. Based on this observation, we have shown that in our stocks there are 20 physical particles to each p.f.u.

Interferon production by single L cells infected with NDV. The microdrop technique has been described in detail (Fleischmann & Simon, 1973). Briefly, L cell monolayers were infected with NDV, and 3 h later, the cells were washed, trypsinized, diluted in super-conditioned media, and dispensed into microdrops at an average of one cell per drop. Four ml of paraffin oil was added to stabilize the drops and prevent evaporation, and those which contained single, live cells were identified microscopically. Next, trypsinized, uninfected L cells were diluted in super-conditioned media to about 2 × 10^5 cells/ml, and approx. 0.015 ml vol. of this dilution were dropped from a finely drawn glass capillary over the previously prepared microdrops. The two drops coalesced, and after allowing 20 min for the cells to settle, the microdrops were again examined, and those containing 30 ± 6 uninfected cells in addition to the infected one were designated as test microdrops. Control microdrops contained only the uninfected cells. The super-conditioned medium, and the relatively large number of cells per microdrop, moderated the rigours of clonal conditions.

Twenty hours later, the microdrops were challenged with 10^5 p.f.u. light sensitive mengoviruses by adding virus with a finely drawn glass capillary. As described below, this gave an effective input multiplicity of 10 p.f.u. cell. It was important to dilute the light-sensitive mengovirus at least tenfold to remove the neutral red, otherwise light-sensitive

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progeny were produced. Three hours post-challenge, the parental virus was light-inactivated and the microdrops were fluid changed to remove both residual virus and neutral red. Progeny mengovirus was harvested at 12 to 14 h post-infection by drawing up the fluid from the microdrops with a glass capillary and depositing the fluid in tubes containing 2 ml of growth medium. This technique removed more than 99% of the virus from the microdrops.

Table 1. Interferon induction by non-infectious haemagglutinin

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Interferon inducer*</th>
<th>NIH⁺ input multiplicity</th>
<th>NDV⁺ input multiplicity</th>
<th>Units of interferon</th>
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<tr>
<td>1</td>
<td>NIH§</td>
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<td>0-01</td>
<td>&lt; 50</td>
</tr>
<tr>
<td></td>
<td>Stock NDV</td>
<td>0-2</td>
<td>0-01</td>
<td>&lt; 50</td>
</tr>
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<td></td>
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<td>5-0</td>
<td>0-25</td>
<td>300</td>
</tr>
<tr>
<td>2</td>
<td>NIH‖</td>
<td>300</td>
<td>0-002</td>
<td>&lt; 6-25</td>
</tr>
<tr>
<td></td>
<td>Stock NDV</td>
<td>4</td>
<td>0-16</td>
<td>100</td>
</tr>
</tbody>
</table>

* L cell monolayers were challenged with 0-2 ml of either stock NDV or NIH isolated from a sucrose gradient of NDV. Supernatant fluids were harvested 24 h post-infection, dialysed at pH 2 for 12 h, and stored at 4 °C.
† Physical particles/cell.
‡ p.f.u./cell.
§ Obtained from a 30 to 50% sucrose gradient with fractions collected from the bottom.
‖ Obtained from a 15 to 30% sucrose gradient with fractions collected from the top. The differences in the number of residual viruses in the NIH samples results from the method of collecting fractions.

RESULTS

Interferon production kinetic curve

Interferon production was first detected 6 h after infection with NDV, with most of it being released between 8 and 12 h. Maximum titres of 1000 to 2000 units/ml were reached at 15 h post-infection and remained constant for at least the next 10 h. Similar results have been reported previously (Paucker & Boxaca, 1967; Thacore & Youngner, 1970).

Efficiency of infection by NDV in L cells and chick embryo cells

In agreement with earlier workers, L cell monolayers infected with NDV at an input multiplicity of 30 produced an average of about 0-3 p.f.u./cell (Paucker, Skurska & Henle, 1962; Lancz & Johnson, 1969; Thacore & Youngner, 1970). Since this yield was too small to cause plaque formation, the synthesis of virus-induced haemagglutinin was used to measure the efficiency of infection in these cells. Using a technique adapted from Ash & Bubel (1966), L cell monolayers were infected with about 100 p.f.u. of NDV/cell. Twelve to 15 h after infection, the monolayer was flooded with chick RBCs, and after allowing 30 min for adsorption at room temperature, the plates were washed and haemadsorption foci counted microscopically. At the same time, plaque formation was determined on CE cells. The number of p.f.u./ml determined in this way exactly equalled the number of haemadsorption centre-forming units; hence, in agreement with Durand & Borland (1969), initiation of infection by NDV was equally efficient in both systems.

Determination of the interferon inducing unit: monolayer studies

When unpurified egg grown NDV was sedimented through a sucrose gradient, two well separated peaks of approximately equal haemagglutinating activity were formed: a rapidly sedimenting peak of infectious material, and a slower peak of non-infectious particles (Dahlberg & Simon, 1966). The latter peak contained little or no RNA (Rott, Reda &
Interferon induction

Fig. 1. Interferon production as a function of input multiplicity. In two separate experiments, interferon production by L cell monolayers infected with NDV at various multiplicities was measured. The empirical data are represented by curve C with separate symbols for each experiment. Three theoretical curves are also presented: Curve A is based on the assumption that maximum interferon production requires infection with 1 or more p.f.u. Curve D is based on the assumption that maximum interferon production requires infection with 2 or more p.f.u. and that there is no production after infection by 1 p.f.u. Curve B is based on the assumption that production of interferon is proportional to input multiplicity up to a maximum of 6 p.f.u. The curves were generated by using the Poisson distribution to calculate the proportion of cells infected by a given number of particles at each multiplicity. For example, under the assumption of curve A, at an input multiplicity of 0.5 p.f.u./cell, 40% of the cells would be infected with 1 or more p.f.u. and hence 40% of the maximum interferon yield would be expected; on the other hand, under this assumption the yield per infected cell would be the same at all multiplicities (see Fig. 2).

Schafer, 1962; J. M. Roman & E. H. Simon, unpublished data), and will be referred to as non-infectious haemagglutinin (NIH). The data presented in Table 1 shows that they did not induce detectable levels of interferon.

While only one tenth of the particles in the infectious peak produced plaques, it was possible that the remaining haemagglutinating particles (hereafter called HAP) could at least induce interferon. To test this hypothesis, the relationship between interferon production and input multiplicity was examined. In Fig. 1, curve C shows that the total amount of interferon produced by a monolayer increased with increasing multiplicity of NDV, reaching a plateau at a multiplicity of 10. Significant levels of interferon were not produced until an input multiplicity of 1 p.f.u./cell was reached, and multiplicities higher than 10 did not
adversely affect interferon production. While these results did not rule out induction of low levels of interferon by HAP, they did indicate that in this system infectious virus was by far the more efficient inducer. Furthermore, a comparison of the theoretical and experimental curves suggests that while one virus can induce interferon, maximum production requires a higher multiplicity.

In Fig. 2, the data of Fig. 1 are replotted as the average amount of interferon produced per infected cell. This value steadily increased, reaching a maximum at an input multiplicity of 10 p.f.u./cell. Again, it is evident that infection with more than one p.f.u. was required to induce maximum interferon production, since, for example, the average yield per infected cell was only 50% of the maximum at an input multiplicity of 1 p.f.u./cell. In these studies, it was not possible to determine whether at low multiplicities all cells produced reduced amounts of interferon, or whether some cells produced maximum amounts while others produced none. Indeed, it was possible that even at high multiplicities only a fraction of the cells produced interferon. To answer these questions, a technique was devised to measure interferon production by single cells.

**Multiplicity of infection for mengovirus-infected L cells in microdrops**

The relatively large fluid vol./cell ratio (30 cells in 0.01 ml in a microdrop, compared to \(4 \times 10^6\) cells in 0.2 ml in a monolayer) made infection in a microdrop very inefficient. To determine the effective multiplicity of a given dose of virus, the yields were determined from microdrops challenged with varying amounts of viruses. All microdrops infected with more than \(10^3\) p.f.u. yielded virus, while at \(10^2\) p.f.u./microdrop, only 30% produced virus. Since virus production by even one infected cell could be detected, only 30% of these microdrops contained an infected cell, and hence the effective multiplicity of \(10^3\) p.f.u. was 0.35 virus particles per microdrop or approx. 0.01/cell. Thus, microdrops were routinely challenged with \(10^3\) p.f.u. virus, giving an effective input multiplicity of 10 p.f.u./cell. This value is sufficient to insure infection of all the cells, but not great enough to overcome the protective effect of low levels of interferon.
Interferon induction

Fig. 3. Interferon production by single L cells infected with NDV at multiplicities of 2.5 and 2.7. Microdrops containing one infected L cell and 30 ± 6 uninfected cells were infected with mengovirus: 12 h later the drops were harvested and the virus assayed (see Methods). The yield per cell in each microdrop was then calculated and expressed as a fraction of the control average and grouped into suitable classes. Microdrops in which the mengovirus yield per cell averaged 0 to 60% of the control were considered to be protected. Control virus yields averaged 255 p.f.u./cell. □, 30 microdrops containing a cell infected at a multiplicity of 2.5; ■, 30 microdrops containing a cell infected at a multiplicity of 2.7; □, 30 control microdrops.

Microdrop studies

Fig. 3 shows the results of typical microdrop experiments. Interferon production was detected by a reduction in the virus yield of test microdrops relative to controls, and microdrops which yielded 0 to 60% of the control average were considered to be protected. Since about 30% of each of the test microdrops, but only 13% of the controls were in this range, some of the L cells must have produced interferon. A similar conclusion is reached by noting the shift away from the 120 to 180% class. Fig. 4 shows the results of an experiment measuring interferon production when multiplicities of 1.7 and 0.17 p.f.u./cell were used. It is clear by the same criteria that a considerable proportion of the L cells from monolayers infected with 1.7 p.f.u./cell produced interferon, but it is doubtful if any of those from monolayers infected with 0.17 p.f.u./cell did.

The data of Fig. 3 and 4 were plotted in a manner that emphasizes the shift of the virus yield to lower values in the presence of NDV-infected L cells. In order to test the effect of the NDV-infected cells more critically, the distribution of each set of protected microdrops was compared with its corresponding control to see if there was indeed a significant difference between them. In this analysis the variance as well as the mean of the sample values were taken into account, and for each paired comparison, a large sample approximate (one sided) normal test was carried out. The results of this analysis are summarized in Table 2, and confirm that many of the NDV-infected L cells conferred protection on the microdrops. The large variances observed with microdrops containing a cell infected with 0.25 or 2.5 NDV/cell are considered in the discussion.
Fig. 4. Interferon production by single L cells infected at multiplicities of 1·7 and 0·17. The experimental procedure was identical to that described for Fig. 3. A total of 120 microdrops were used: ■, 35 control microdrops; □, 35 microdrops infected with NDV at a multiplicity of 1·7; □, 50 test microdrops infected with NDV at a multiplicity of 0·17. Control virus yields averaged 206 p.f.u./cell.

The foregoing analysis showed that interferon production was a common event; however, to calculate the frequency of interferon producing cells at each multiplicity it was necessary to correct for the control microdrops which yielded in the 0 to 60 % range.* This was done by means of the relation:

\[
NP_{\text{act}} = \frac{NP_{\text{obs}}}{NP_{\text{con}}} 
\]

where \(NP_{\text{act}}\) = actual percentage of non-protected test microdrops, \(NP_{\text{obs}}\) = observed percentage of non-protected test microdrops, \(NP_{\text{con}}\) = observed percentage of control microdrops in the non-protected range. The actual percentage of protected microdrops in any experiment is then equal to \(100 - NP_{\text{act}}\). By determining the actual percent protected cells for each experiment, this correction permits the results of separate experiments to be compared. Table 3 gives both the observed and the corrected percentages of interferon producing cells for several microdrop experiments (including those shown in Fig. 3 and 4), representing almost 400 single cells. At least 50 % of the test microdrops were protected at a multiplicity of 25. If the microdrop technique is considered an infectious centre assay which measures interferon production rather than virus yield, then the observed 50 % efficiency compares favourably with other infectious centre assays as reported in the literature: 16 to 20 % for the Sindbis virus-CE cell system (Stewart & Sheaff, 1969), 38 to 50 % for Western equine encephalomyelitis virus-L cell system (Lockart & Horn, 1963) and 35 to 87 % for the mengovirus-L cell system (Brownstein & Graham, 1961). Hence, it is likely that the ability to produce interferon is a trait common to all, or nearly all, the cells in the culture.

In Table 4, observed and predicted frequencies of interferon producing cells are compared.

* It is incorrect to merely subtract the percentage of control microdrops from the percentage of test microdrops in the protected range, as can be readily seen in the case of complete protection by interferon.
Interferon induction

Table 2. Yield of mengovirus from microdrops protected by NDV-infected L cells

| Expt. | Approximate input multiplicity* | Z|| |
|-------|-------------------------------|----|
|       | 0 (Control)                   | 0.25 | 2.5 | 25 | |
| 1     | I X 54.1 (8)‡†               | 33.0 (17) | 1.88‡ |
|       | 2X§                          | 231  | —   | —  | |
| 2     | X 61.45 (20)                 | 15.1 (48) | 5.03** |
|       | S 428                        | 643  | —   | —  | |
| 3     | X 83.9 (26)                  | 44.6 (52) | 4.25** |
|       | S 752                        | 959  | —   | —  | |
| 4     | X 66.0 (27)                  | 83.3 (55) | —   | —  | |
|       | S 540                        | 1521 | —   | —  | |
| 5A†† | X 97.8 (28)                  | 49.1 (31) | —   | —  | Not significant |
|       | S 2868                       | 3881 | —   | —  | |
| 5B    | X 97.8 (28)                  | 66.2 (31) | —   | —  | 2.02¶ |
|       | S 2868                       | 4417 | —   | —  | |
| 6A    | X 254.8 (30)                | 197.7 (39) | —   | —  | 2.2¶ |
|       | S 7859                      | 11839 | —   | —  | |
| 6B    | X 254.8 (30)                | 169.2 (39) | —   | —  | 2.7** |
|       | S 7859                      | 6043 | —   | —  | |
| 7A‡‡ | X 110.9 (35)                 | 136.8 (50) | —   | —  | Marginal |
|       | S 7095                      | 11936 | —   | —  | |
| 7B‡‡ | X 110.9 (35)                 | 112.1 (35) | —   | —  | Not significant |
|       | S 7095                      | 17356 | —   | —  | |

* NDV/L cell.
† Average yield of mengovirus/L cell.
‡ Number of microdrops.
§ Sample variance.
|| Z = \frac{X_1 - X_2}{\sqrt{(S_1^2/n_1 + S_2^2/n_2)}}, where X_1 and X_2 are the two sample means; S_1^2, S_2^2 are the two sample vari-
ances, and n_1, n_2 are the respective sample sizes.
¶ Significant at < 5% (one sided test).
** Significant at < 1% (one sided test).
†† Significant but in 'wrong direction'.
‡‡ Series A and B used the same control values.

The predicted frequencies are based on the assumptions that (a) the 10:1 HAP:p.f.u. ratio
derived from our haemagglutination studies is correct, and (b) that either 1 HAP or 1 p.f.u.
is sufficient to induce maximum interferon production. Since at a multiplicity of 0.25 p.f.u./
cell the results are in gross disagreement with the values predicted if HAP can induce inter-
erferon, it would appear that in this system the only particles that can induce are those capable
of carrying out a productive infection.

The observed levels, however, are somewhat lower than expected if infection of a cell
with one p.f.u. invariably induces maximum interferon production. The efficiency of induc-
tion can be estimated by dividing the observed frequency of protected cells by the expected
values, as shown in Table 5. The resulting figures are in striking agreement with the relative
yield of interferon obtained from monolayer cultures infected at the identical multiplicities
(see Fig. 2). Thus, it appears that at an input multiplicity of 0.25, only 42% of the infected cells
produce interferon. This implies that infection with a single plaque-forming particle can
induce interferon, but that the probability of induction depends on the number of particles
participating in the infection.
Table 3. *Summary of interferon production by single NDV-infected L cells.*

<table>
<thead>
<tr>
<th>Approximate input multiplicity</th>
<th>Expt.</th>
<th>No. of microdrops</th>
<th>Observed % microdrops in protected range (0–60% control average)</th>
<th>% Protected test microdrops</th>
<th>Weighted average¶</th>
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* The experimental procedure is the same as for Fig. 4. A total of 388 test microdrops are represented: 154, 97, and 137 at multiplicities of 25, 2:5, and 0:25 p.f.u./cell, respectively. Control yields ranged from 54 to 255 p.f.u./cell for the separate experiments.

†, ‡, §. Same symbols designate parts of the same experiment.

¶ The procedure for calculating the percentage of protected microdrops is given in the text.

Σ Microdrops x % protected test microdrops
Σ microdrops

Table 4. *Summary of interferon production by single L cells. II*

<table>
<thead>
<tr>
<th>Input multiplicity (p.f.u.)</th>
<th>Observed†</th>
<th>Corrected‡</th>
<th>Predicted§</th>
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<td>100</td>
<td>100</td>
</tr>
<tr>
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<td>69</td>
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<tr>
<td>0:25</td>
<td>48</td>
<td>9:2</td>
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* See Fig. 4 for experimental procedures and Table 2 for the data.
† See Table 2.
‡ Obtained by normalizing the observed values to an assumed efficiency of 52% (see text).
§ See text.

Table 5. *Summary of interferon production by single NDV infected L cells. III*

<table>
<thead>
<tr>
<th>Input multiplicity (p.f.u.)</th>
<th>Corrected % producing cells†</th>
<th>Predicted % producing cells‡</th>
<th>Cellular efficiency‡ (%o)</th>
<th>Monolayer efficiency§ (%o)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2:5</td>
<td>69</td>
<td>92</td>
<td>75</td>
<td>72</td>
</tr>
<tr>
<td>0:25</td>
<td>9</td>
<td>22</td>
<td>42</td>
<td>34</td>
</tr>
</tbody>
</table>

* See Fig. 4 for the experimental procedures.
† See Table 3.
‡ Obtained by dividing column 2 by column 3. See text for details.
§ See Fig. 2.
DISCUSSION

It is well known that artificially inactivated viruses are capable of inducing interferon (see Introduction), but it is unclear if naturally occurring non-plaque-forming particles can also do so. The NDV-L cell system was used to investigate this question. NDV induces high titres of interferon in L cells, and its stocks contain three classes of particles which can be tested for interferon inducing ability: (1) plaque-forming haemagglutinating particles (p.f.u.) (~ 5%); (2) non-plaque-forming haemagglutinating particles containing RNA (HAP) (~ 45%) and (3) non-plaque-forming haemagglutinating particles which contain little or no RNA (NIH) (~ 50%).

NIH, as purified from a sucrose gradient, are incapable of inducing interferon (Table 1). While it is not certain if these particles are completely free of RNA, it is sufficient for our purposes to note that in this system they cannot be responsible for induction of appreciable amounts of interferon. Induction by HAP is a more difficult question. A separate class of HAP is inferred from the fact that only 10% of the particles containing RNA form plaques. It is not clear, however, if these particles are inherently different from the p.f.u. It is possible, for example, that all particles containing RNA have an equal probability of initiating infection, but only about 10% succeed. Nevertheless, those particles which do not carry out a productive infection, for whatever reason, may still be able to initiate interferon production. This question was approached by studying the effect of input multiplicity on interferon production by monolayers and single cells. In monolayers, maximum interferon production was reached at multiplicities of 10 to 300 p.f.u./cell, while at a multiplicity of 5 HAP per cell (0.5 p.f.u./cell) only about 16% of the maximum interferon production was observed (Fig. 1, 2). The estimate of 10 HAP/p.f.u. is based on Isaacs (1957) determination that, at the haemagglutination endpoint, there is one physical particle/red blood cell; any error would appear to be in the direction of increasing the HAP/p.f.u. ratio and would make the results even more striking. Hence, by comparing the experimental and theoretical curves in Fig. 1 and 2, three conclusions can be drawn from these data: (1) high multiplicities of NDV did not inhibit interferon production; (2) more than one plaque-forming particle per cell was needed for maximum production, but one particle can produce some interferon and (3) non-plaque-forming particles (HAP) are, at best, weak inducers of interferon.

A microdrop technique was used to extend these observations and to answer the important question: what proportion of the infected cells actually produce interferon? About 30 uninfected cells were added to one NDV-infected cell and subsequently infected with mengovirus. By measuring the yield from these cells, it was possible to determine if the original NDV-infected cell had produced interferon. It is clear that any effect must be due to interferon or a similar substance rather than virus interference since the yield from the NDV-infected cell is less than one p.f.u.; furthermore, absorption of any released virus particles would be extremely inefficient. Since $2 \times 10^6$ L cells usually produce about 8000 units of interferon (4 ml at 2000 units/ml) or $4 \times 10^{-3}$ units per cell and the vol. of the microdrop was about 0.01 ml, the effective concentration in the microdrop was 0.2 to 0.5 units/ml, or close to the limit of detectability. To maximize the sensitivity of the assay, we used the fact that the primary effect of low levels of interferon is to delay the time of virus burst (Fleischmann & Simon, 1973) and measured the yield of test and control microdrops 12 to 14 h after infection. Considering the marginal production of interferon by individual cells, the relatively weak degree of protection was not surprising. Nevertheless, Fig. 3 and the data of Tables 2 and 3.
show that at a multiplicity of 25 p.f.u./cell, two to four times more test microdrops were in
the protected range than control microdrops, and hence interferon production is a property
common to many cells. This is the major conclusion we wish to draw; however, a more
detailed analysis of the data is of interest. The data of Table 2 shows that while the variance
of virus yields from microdrops containing L cells infected with 25 p.f.u. of mengovirus is
smaller than the controls (because of selective loss of the higher yielding drops), microdrops
containing L cells infected with 0.25 or 2.5 p.f.u. of NDV tend to have a larger variance.
Two explanations may be offered: (1) Since only a fraction of cells yield interferon at the
lower multiplicities two distinct populations of microdrops are involved; protected and non-
protected. Furthermore, some L cells may lyse without yielding interferon and this may
enhance the mengovirus yield from the surviving cells (N. A. Miner & E. H. Simon,
unpublished observations) and account for the enhanced yields sometimes seen from
‘protected’ microdrops (Table 2, cases 4 and 7A). (2) In addition to protected and non-
protected populations, low levels of interferon may actually enhance virus yield. We have
consistently observed a slightly higher efficiency of plating of mengovirus on monolayers
protected with limiting amounts of interferon.

As shown by the data in Table 3, about 50% of the single cells produced interferon. It is
difficult to determine if this value is a measure of the efficiency of the technique or an absolute
measure of the capacity to produce interferon. However, if the microdrop technique is
viewed as an infectious centre assay which uses interferon production rather than plaque
formation as a measure of infectivity, its 50% efficiency compares favourably with values
obtained by other methods (Brownstein & Graham, 1961; Lockart & Horn, 1966; Stewart &
Sheaf, 1969). Viewed in this manner, it seems likely that in this system essentially every cell is
capable of producing interferon.

Since, as shown in Table 4, only 9.2% of the cells produced detectable interferon at a
multiplicity of 2.5 HAP/cell (0.25 p.f.u./cell), it is apparent that, at least in this system,
viruses which do not carry out a productive infection induce little, if any, interferon. The
observations that HAP do not appear to induce interferon suggest that u.v.-inactivated NDV
and naturally occurring non-infectious particles from NDV stocks interact with the cell in
basically different ways. Alternatively, it may be that only 1 in 10 of the HAP actually
succeeds in establishing a functioning genome within the cell, with the rest being rapidly
eliminated by cellular defences. (A situation roughly analogous to that which occurs with
poliovirus (Joklik & Darnell, 1961)). In any event, the efficiency of induction of interferon in
single cells by active virus is essentially identical to the efficiency of production of interferon
in a monolayer when the same input multiplicities were used (Tables 3 and 4). Taken together,
these results indicate that a single p.f.u. can induce interferon production, but that to insure
maximum, or perhaps any production by some cells, infection with more than one plaque-
forming virus is needed.

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Interferon induction


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