Interferon Production by Individual L Cells

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SUMMARY

Using a protected centre technique in which agarose prevents the diffusion of interferon from individual producing cells, we have shown that essentially every cell in a monolayer of mouse L cells can be induced to produce interferon by infection with Newcastle disease virus (NDV). The amount of interferon produced by individual cells appeared to be highly variable, even when cloned cells and viruses were used. U.v.-irradiated virus lost its capacity to induce interferon in L cells and to infect chick embryo fibroblasts at the same rate. A small proportion of cells (1 × 10⁻⁶ to 10 × 10⁻⁶) appeared to produce interferon constitutively. This fraction was increased threefold by u.v. irradiation of the cells, and up to 10-fold by exposing cells to the mutagen ethyl methane sulphonate.

INTRODUCTION

Interferon induction and production are usually investigated in terms of the average response of large populations of cells (see for example, Ho & Armstrong, 1975) and data for the amounts of interferon produced by individual cells in response to different inducers are lacking. Such data could answer a number of important questions. For example, does priming (Billiau, 1970; Rosztóczy & Mécás, 1970) superinduction (Vilcek & Ng, 1971) or a ‘good’ inducer boost the yield of each individual cell, or does it increase the proportion of producing cells? Data on interferon production by individual cells would facilitate the isolation of high interferon producers, investigation into the mechanisms and genetics of interferon induction and production, and the detection of cells which constitutively produce interferon.

Previous single cell studies (Fleischmann & Simon, 1974; Rogers & Merigan, 1974) indicated that as many as 50% of the cells in a population could be induced by virus infection to produce interferon. Osborn & Walker (1969) and Kronenberg (1977) developed techniques whereby interferon production from individual cells could be detected directly. Induced cells were suspended in agarose and added to a monolayer of sensitive cells. The retained interferon induced a discrete centre of protected cells which was revealed after infection and subsequent staining of the monolayer. We have independently developed a simpler and more sensitive protected centre assay, utilizing is-1 (an interferon-sensitive mutant of Mengovirus) as the challenge virus. This has allowed us to show that while every mouse L cell can be induced to produce interferon by infection with a single infectious Newcastle disease virus (NDV) particle, non-infectious particles apparently do not induce.

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We have consistently observed cells that constitutively produced interferon in our cultures. However, both the proportion of such cells and the amount produced by each cell varied widely. Exposure of the cells to u.v. irradiation or to the alkylating agent ethyl methane sulphonate (EMS; Loveless, 1958) markedly increased the fraction of surviving cells which constitutively synthesized interferon.

METHODS

Cells, viruses and anti-interferon antibody. An L cell line maintained in our laboratory for 10 years and a KB line obtained from Arthur D. Little Inc. (Boston, Mass.) were grown in a humidified CO₂ incubator at 37 °C in minimum essential Eagle’s medium supplemented with 10% new-born calf serum (medium) as described previously (Fleischmann & Simon, 1974). Cells were cloned by diluting them to 1 cell/ml and distributing 0.1 ml aliquots into Microtitre trays (Carlson Scientific Inc., Peotone, Ill.). Five to ten days later, clones which appeared to be derived from single cells were selected and grown into stocks.

NDV (Beaudette strain) was cloned by injecting fluid from individual plaques grown on chick embryo fibroblasts (CEF) monolayers into 11-day-old embryonated eggs. This procedure was repeated three times. For u.v. irradiation, 1 ml samples of virus diluted in phosphate buffered saline solution (PBS; Dulbecco & Vogt, 1954), were exposed in 60 mm disposable plastic tissue culture dishes to a General Electric G15T8 germicidal bulb at a distance of 43 cm for varying times; the dose rate was 26 ergs/mm²/s but the efficiency of inactivation varied with the extent of dilution into PBS.

Mengovirus is-i is a strain which is 20 to 100 times more sensitive to interferon than wild-type virus in the line of L cells used in these experiments (Simon et al. 1976).

Purified antibody to mouse interferon was obtained from the National Institute of Allergy and Infectious Diseases (NIAID) and resuspended in sterile saline at 3000 units/ml. It was stored at −20 °C until use.

Assay for interferon production by individual monolayer cells by the protected centre technique. This procedure is similar to that of Osborn & Walker (1969); 0.2 ml vol. of NDV diluted in PBS:medium (1:1) (this diluent gave lower backgrounds than either PBS or medium alone) were plated on confluent 24-h L cell monolayers (derived from uncrowded 150 mm stock plates) in 35 mm dishes. After incubation for 1 h, the inocula were removed. The monolayers were rinsed twice with medium at 37 °C, overlaid with 0.4% agarose (United States Biochemical Corp., Cleveland, Ohio) in medium and incubated for 30 to 40 h. The agarose overlay was then removed and the monolayers were rinsed with warm medium to remove all traces of the agarose, then challenged with is-i virus at an m.o.i. of 4 to 10 in 0.2 ml medium. After 1 h at 37 °C, 1 ml of medium was added. The cultures were incubated for an additional 12 h and were then stained with 0.2% neutral red for 30 min. Protected centres appeared as stained areas of surviving cells surrounded by unstained dead cells. The protected centres could also be visualized by the use of Giemsa or trypan blue stains or by microscopic observation without staining. In certain experiments, the protected centres were produced without an agarose overlay. The resulting centres tended to be larger and more diffuse, but in moderate numbers could still be counted accurately. As we will show, the protected centres represent areas where NDV infected cells induced interferon synthesis.

Protected centre technique using infective centres. After infection with NDV, as described above, the inocula were removed and the monolayer rinsed three times with medium. Two ml of medium were added and the plates incubated for an additional 2 to 3 h. The cells were then rinsed twice with PD (PBS without divalent cations) and trypsinized with 0.05% (w/v) trypsin in PD at 37 °C for 3 min. The dispersed cells were sedimented three times in medium, diluted in medium and counted in a haemocytometer; 0.2 ml of cell
suspension was allowed to adsorb to uninfected monolayers for 30 min. The medium was carefully aspirated and the monolayers covered with 0.2 ml of 0.4% agarose in medium which was then allowed to harden at room temperature for 15 min before the addition of a further 2 ml of agarose in medium. The assay was then continued as above.

**Induction of protected centre by u.v.-irradiation.** L cells (2 × 10^6) in 2 ml of medium were incubated in 35 mm dishes for 18 to 24 h. The monolayers were rinsed three times with PBS, overlaid with 1 ml PBS and irradiated for varying times with a General Electric G15T8 germicidal bulb at a distance of 70 cm (dose rate: 10 ergs/mm²/s). After removal of the PBS, the cells were overlaid with 2 ml 0.4% agarose in medium and the assay was continued as above.

**Cell survival following u.v.-irradiation.** L cells (200 and 2000) were placed in separate 60 mm dishes containing 5 ml medium and 40 μg/ml DL serine and incubated for 18 h. The monolayers were then irradiated as above. The cells were aspirated, overlaid with medium supplemented with 40 μg/ml DL serine and incubated for an additional 7 days. Colonies containing 8 or more cells were counted. The cloning efficiency for the unirradiated control cells was about 30% and this was presumed to represent 100% survival.

**Ethyl methane sulphonate (EMS) induction of protected centres.** Cells (10^5) in 5 ml of medium were incubated in 60 mm dishes for 3 h. EMS (Eastman, Rochester, N.Y.) was then added and the cells incubated for an additional 21 h. After rinsing, the cells were overlaid with 4 ml medium and incubated again for 24 h. After removal of the medium, 4 × 10^6 untreated cells in 4 ml medium were added and incubated for 3 to 6 h to allow for attachment. The monolayers were then aspirated and overlaid with 5 ml agarose in medium and the protected centre protocol continued as described above.

**Cell survival following treatment with EMS.**

**Cell growth studies.** Cells (40 000) in 2 ml medium were placed into 35 mm dishes and incubated for 3 h. EMS was added and the cells incubated for an additional 21 h. The EMS was removed and the cells were overlaid with 2 ml medium supplemented with 40 μg/ml DL serine (cloning medium) and re-incubated. The cells within 1 mm circles (previously marked on the bottom of the dishes) were counted at the time the EMS was removed (zero time) and at 24 h intervals thereafter.

**Cloning efficiency.** Cells (500 to 500 000) in 4 ml medium were placed into 60 mm dishes and treated as described above, except that after the EMS was replaced with cloning medium the cells were not disturbed for 11 days. At that time the clones were fixed with 10% formaldehyde, stained with Giemsa, and counted.

**Haemadsorbing centres.** Chicken red blood cells were used for the detection of NDV infected cells as described by Marcus & Carver (1969).

**Assay of anti-interferon antibody.** Five units of interferon at a concentration of 20 units/ml were mixed with 0, 1·5, 3, 4·5 and 6 units of antibody (as defined by NIAID); 0·05 ml of each mixture was added to each of five wells in a Microtiter tray containing 2 × 10^4 L cells/well. Control wells containing antibody only and medium only were also included. After 24 h incubation, the test wells were each infected with 100 p.f.u. of is-1 virus and scored daily for c.p.e. for 3 days.

**Effect of anti-interferon antibody on protected centres.** Preliminary studies showed that adding moderate amounts of antibody to the agarose overlay did not inhibit production of spontaneous or induced protected centres. However, positive results were obtained with the following technique: 35 mm plates were prepared with 8 × 10^6 L cells and infected with NDV 24 h later. After 1 h the plates were overlaid with either 1 ml medium or medium plus antibody. Thirty-six h later the medium was removed and the plates infected and counted as described above.
RESULTS

Protected centre induction by infectious NDV

Fig. 1 is a photograph of protected centres (mock infected on the left, 100 p.f.u. NDV on the right) stained with Giemsa. Although some centres were found if the agarose overlay was left on for as little as 12 h, 30 to 40 h was optimal. There was a close correspondence between the number of protected centres appearing in an L cell monolayer and the number of infectious NDV (measured on CEF) used to induce those centres (Fig. 2). The efficiency of induction ranged between 60 and 100%.

There was considerable variation in the sizes of the protected centres. This intrinsic variation remained even when any of five freshly cloned cell lines or any of four triply cloned NDV stocks were used. To eliminate complications due to any possible contaminating chicken viruses, NDV was picked from plaques on KB cell monolayers and used directly to induce protected centres. The variation remained. The effect of spread of the NDV infection on protected centre size was investigated by haemadsorption with chick red blood cells. Monolayers not exposed to NDV did not have haemadsorbing cells, while monolayers infected with NDV contained about 20%, more haemadsorption centres than expected on the basis of virus titre. Although there were often several haemadsorbing cells in a single haemadsorbing centre, some of the largest protected centres contained only a single such cell. The yield of NDV from our line of L cells never exceeded 0·1 p.f.u./cell and we feel that the excess of haemadsorbing cells was due, at least in part, to non-specific spread of NDV antigen following removal of the agarose overlay.
Fig. 2. Correlation between the infectious titre and protected centre titre of NDV as measured on chick embryo fibroblasts and mouse L cells, respectively. The protected centre values have been corrected by subtracting the background of two centres per plate found on mock infected cultures.

In monolayers not exposed to NDV, 'spontaneous' protected centres were found at a frequency of $1 \times 10^{-6}$ to $10 \times 10^{-6}$ per cell. However, within a given experiment, the number of protected centres was normally distributed about its mean. Subsequent work showed that a major cause of the experimental variation was the condition of the stock plates from which the test monolayers were derived, with subconfluent stock plates giving rise to test plates with the lowest backgrounds.

The protected centres (both induced and spontaneous) could be kept alive indefinitely if, following inoculation with Mengovirus, the cells were overlaid with agarose containing antiserum against Mengovirus and 200 units/ml interferon and incubated for several days, after which the surviving cells could be grown to confluency in normal medium containing antibody and interferon and passaged indefinitely. However, even after 3 weeks of subculture, the cells succumbed to Mengovirus infection when antibody and interferon were removed. In the case of NDV-induced centres, haemadsorbing cells disappeared following several days of growth in the presence of antiserum against Mengovirus and interferon.

Assay of anti-interferon antibody

Table 1 presents the combined results of two experiments designed to measure the ability of antibody against interferon to inactivate it under conditions similar to those present during protected centre formation. The last line shows that antibody did not have a toxic effect on the cells and the results indicate that under these conditions 3 units of antibody inactivated 5 units of interferon.

Effect of anti-interferon on protected centre formations

The data of Table 2 show the effect of 36 units of antibody on spontaneous and NDV-induced protected centres. In repeated experiments, protected centres formed in the presence of antibody were fewer and less than $1/3$ the size of the one produced in its absence. At higher levels of NDV (300 or more p.f.u./plate) control plates were completely protected, while treated plates showed discrete protected centres. This is consistent with antibody reducing the spread of interferon from the producing cells. Higher levels of antibody probably would have completely eliminated the protected centres, but we did not have enough antibody to test this. The low efficiency of protected centre formation shown in Table 2 was probably caused by the absence of an agarose overlay and the consequent loss of small centres.
Table 1. Inactivation of interferon by antibody*

<table>
<thead>
<tr>
<th>Antibody (units)</th>
<th>Virus</th>
<th>Interferon</th>
<th>Fraction of wells with 100% c.p.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0/10</td>
</tr>
<tr>
<td>1.5</td>
<td>+</td>
<td>+</td>
<td>6/10</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>10/10</td>
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<tr>
<td>4.5</td>
<td>+</td>
<td>+</td>
<td>10/10</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>10/10</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* Five units of interferon were mixed with varying quantities of antibody and added to 2 x 10^4 L cells in a Microtiter tray. The maximum c.p.e. was seen after 3 days. Details are given in Methods.

Table 2. Inhibition of protected centre formation by anti-interferon antibody*

<table>
<thead>
<tr>
<th>Antibody (units)</th>
<th>NDV (p.f.u./plate)</th>
<th>Number of plates</th>
<th>Number of protected centres per plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>6</td>
<td>4.7</td>
</tr>
<tr>
<td>36</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>36</td>
<td>50</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>150</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>36</td>
<td>150</td>
<td>2</td>
<td>10</td>
</tr>
</tbody>
</table>

* Plates were infected with the indicated p.f.u. of NDV and overlaid either with control medium or medium containing antibody. Protected centres formed in the presence of antibody were less than 1/3 the size of those on the control plates.
† Three units of antibody inactivated 5 units of interferon under the conditions used.

Lack of protected centre induction by non-infectious virus

An L cell exposed to a single naturally occurring non-infectious particle did not produce a detectable level of interferon since the number of protected centres did not exceed the virus titre on CEF plates. The question of induction by non-infectious virus was further investigated using u.v.-inactivated NDV. In this experiment, the ability of irradiated virus to form plaques on CEF or protected centres on L cells was compared. The data of Fig. 3 show that only infectious particles appeared to be capable of inducing interferon. This experiment also indicated that the variation in protected centre size was not due to virus aggregates, since the inactivation followed the single-hit kinetics expected of single particles.

Our finding that u.v.-inactivated NDV did not induce interferon in L cells conflicts with other data (Cantell & Paucker, 1963). However, since these investigators infected cells at high multiplicities while our cells were never exposed to more than a single physical particle, it seemed possible that amplification of some virus product is needed for interferon induction; such amplification could be provided either by productive infection or by exposure to a large number of virus particles. This possibility was investigated by infecting monolayers at an m.o.i. of either 0.05 or 20 (based on the titre before u.v.-irradiation) and measuring the ability of these cells to induce protected centres when overlaid on non-infected cells (infected centre assay). The data, presented in Table 3, show that all of the observed protected centres could be accounted for by infectious virus. In addition, there was no significant difference in the sizes of the protected centres between this experiment and the experiments described in Fig. 1 and 3.
**Interferon from single cells**

![Graph showing capacity of u.v.-irradiated NDV to induce protected centres](image)

**Fig. 3.** Capacity of u.v.-irradiated NDV to induce protected centres. The protected centre values have been corrected by subtracting the background of 2 centres per plate found on mock-infected cultures. □, Protected centres; ○, plaques on CEF.

**Table 3.** Protected centre formation by untreated and by u.v.-inactivated NDV using infective centres

<table>
<thead>
<tr>
<th>NDV surviving fraction*</th>
<th>M.o.i.†</th>
<th>Expected</th>
<th>Observed</th>
<th>Efficiency§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>147</td>
<td>26</td>
<td>0.18</td>
</tr>
<tr>
<td>1</td>
<td>0.05</td>
<td>165</td>
<td>25</td>
<td>0.15</td>
</tr>
<tr>
<td>3.5 × 10⁻⁴</td>
<td>20</td>
<td>714</td>
<td>136</td>
<td>0.19</td>
</tr>
<tr>
<td>3.5 × 10⁻⁴</td>
<td>0.05</td>
<td>4</td>
<td>1</td>
<td>0.25</td>
</tr>
</tbody>
</table>

* Ratio of the infectious titre of NDV following u.v. irradiation to that in the absence of u.v.
† Multiplicity of infection based on titre in the absence of u.v.
‡ Expected protected centres: the number of centres/plate which would result if only infectious NDV could induce them and if every infected cell which was overlaid on the test monolayer resulted in a protected centre. The values for observed protected centres are the average of 4 plates/data point. The mock-infected controls yielded 1 centre/plate. Inocula (no inactivation and m.o.i. 20) from which the cells had been removed by centrifugation or which had been frozen-thawed three times resulted in values more than 100-fold less than that shown in the table.
§ Efficiency is the ratio of observed to expected protected centres.

**Induction of protected centres by u.v. and EMS**

The presence of protected centres in uninfected monolayers suggested the existence of cells that were genetically constitutive for interferon production, and that their frequency might be increased by treatment with a mutagen such as u.v.-irradiation. The results of such an experiment demonstrated (Fig. 4a) that treatment with u.v. increased the frequency of protected centres, but at a much slower rate than it killed cells. Thus, induction of protected centres by u.v. was not an invariable consequence of cell death. The data in
Fig. 4. U.v. irradiation induces protected centres more slowly than it kills L cells. Monolayers of L cells were irradiated at 10 ergs/mm²/s. Cell survival and the induction of protected centres were measured as described in Methods. (a) ○—○, Protected centres per 10⁶ cells; □—□, % cell survival. (b) The data from (a) was re-calculated to give protected centres/10⁶ surviving cells.

A second mutagen, ethyl methane sulphonate (EMS), was also studied. To eliminate the possibility that EMS merely sensitized cells to pre-existing interferon, subconfluent plates were treated with EMS and sufficient untreated cells were added to form a monolayer. Thus, most of the surviving cells in the protected centres were never exposed to EMS. The data in Fig. 5(a) show that there was a five- to tenfold increase in the absolute number of protected centres following treatment with EMS. In addition, the number of surviving cells as a function of EMS dose was measured both by cell growth analysis and cloning efficiency. The data are replotted in Fig. 5(b) to give the number of protected centres relative to the number of surviving cells. The results show a striking increase in interferon-producing cells.
Fig. 5. Induction of protected centres by EMS. (a) Protected centres/10⁴ treated cells. A background of 6 centres/plate has been subtracted from the data. (b) Protected centres/10⁶ treated and surviving cells. The data in (a) have been re-plotted on the basis of the cell survival information given in the insert (c) which shows cell survival following exposure to EMS. Survival was calculated both from the ability of the cells to grow in monolayers (●—●) and from their capacity to form colonies (○—○). The growth rate (generation time of 22 h) and the cloning efficiency (40%) of untreated cells were assumed to represent populations in which every cell was a "survivor".

DISCUSSION

The protected centre technique was used to measure the number of interferon-producing cells in a monolayer. Within the limits investigated, the number of protected centres increased linearly with the NDV dose, and both induced and spontaneous centres had a similar appearance and size range. Protected centres were clearest when the agarose overlay was present for 30 to 40 h, a time consistent with that required for the induction, production,
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release and action of interferon. Presumably the overlay allowed locally high interferon concentrations to accumulate. In some experiments, protected centres were induced without the use of an overlay. As expected, these were larger and more diffuse than the ones developed under agar and, consequently, the modified assay was less sensitive than the standard one.

The low yield of NDV (less than 0.1 p.f.u./cell from cultures infected at an m.o.i. of 10) made it an ideal virus inducer for these experiments. The presence of large protected centres containing only one cell with NDV haemagglutinating antigen (HA) on its surface and the observation that the great majority of cells within the protected centres did not express HA, indicated that there was little, if any, spread of the NDV infection beyond the initial cells. In addition, these observations showed that the NDV infection could not have produced the centres by directly interfering with Mengovirus replication, and that apparently, NDV (and the other treatments) induced an interferon-like substance which then protected the surrounding cells. This presumption was confirmed by showing that antibody against interferon reduced both the size and the number of protected centres whether they were induced by NDV or were spontaneous. However, elimination of protected centres required more antibody than expected (compare Tables 1 and 2). Apparently the concentrations of interferon in the vicinity of a producing cell can be quite high and large amounts of antibody are needed to inactivate it. In addition, it is possible that cells in contact with the producing one may be protected by direct passage of the interferon molecule through the cell membrane. This may explain why it was easier to demonstrate a reduction in the size of induced protected centres than an absolute decrease in their number. On the other hand, spontaneous centres, being smaller to begin with, were eliminated completely (Table 2).

While some cell-to-cell spread of NDV might have occurred, (as indicated by the haemadsorption results), there was no clear correlation between the number of haemadsorbing cells associated with a protected centre and its size. Detailed interpretation of these results is difficult because HA may be either lost or transferred to other cells following removal of the agarose.

The basic limitation of the assay is the sensitivity of the challenge virus to the interferon-induced antiviral state. Hence it was preferable to use the is-1 mutant of Mengovirus as a challenge virus rather than the more interferon-resistant wild type. Similarly, vesicular stomatitis virus was completely unsuitable as a challenge virus because the local concentrations of interferon did not prevent cell death.

The data indicated that virtually every L cell can be induced to produce interferon following infection with a single infectious NDV particle. Similarly, every infectious NDV particle was capable of inducing interferon. Since there are ten non-infectious physical particles per infectious particle in an NDV stock and half of them contain RNA (Dahlberg & Simon, 1969; Roman & Simon, 1976), it was anticipated that at least some of the non-infectious particles might be capable of inducing interferon. However, neither they nor the u.v.-inactivated virions appeared to do so (since monolayers were routinely scanned with a dissecting microscope, even very small protected centres would have been detected).

The inability of the centres to be induced by naturally occurring non-infectious NDV particles may be related to the fact that a heterologous host was used. Since u.v.-inactivated NDV is a more effective interferon inducer than infectious NDV in the 'natural' NDV/chick cell system, chick cells might provide a better study system.

The inability of u.v.-inactivated NDV to induce protected centres in the L cell system is in conflict with studies on bulk interferon production by others (Cantell & Paucker, 1963). However, direct comparisons are difficult since such studies have generally involved
unknown amounts of residual infectious virus, exposure of the cells to extremely high m.o.i. of virus (based on initial titre) and different strains of cells and virus.

Presumably, the size of a protected centre is related to the amount of interferon produced and the large variation in centre sizes (up to 100-fold in surface area) observed under all conditions (including induction by NDV, u.v.-irradiation, exposure to EMS and apparent spontaneous induction) suggested that there may exist considerable genetic variation in the capacity of individual cells to produce interferon. We could not confirm this in respect to virus induction since centres induced by triply cloned NDV in freshly cloned L cells also showed variation in size. However, since at least 30 cell divisions intervened between isolation of a single cell and execution of the experiment, genetic variation cannot be excluded. Alternatively the variation might be due to metabolic heterogeneity resulting from either induction of the cells at different stages of the cell cycle, or to local conditions of crowding on the monolayers.

The system is unsuitable at present for determining the basis for superinduction and priming. It is difficult to superinduce L cells (Weber et al. 1976) and our attempts to apply the system to superinducible human fibroblasts have been hampered by their unsuitable morphology (the very elongated cells gave diffuse centres), the lack of a suitable challenge virus (is-1 kills human FS-4 and FS-7 cells, even in the presence of high doses of interferon; G. E. Brown, unpublished data) and the relatively low interferon yields from such cells (Billiau et al. 1972). Thus during superinduction the yield per producing cell rather than the number of producing cells may be increased, a conclusion that can also be drawn from studies on protected centre formation with human cells (Kronenberg, 1977).

The protected centres found in the absence of known inducers appear to represent spontaneous interferon production by individual cells for the following reasons. First, the centres are similar in size and appearance to those induced by NDV. Second, their symmetrical form indicates that they are not due to the presence of residual agarose on the assay plates or loss of virus receptors during removal of the agarose. Third, a subline of L cells was found which did not exhibit spontaneous protected centres. Fourth, and most conclusive, their production was eliminated by treatment with anti-interferon serum. Thus, it is highly unlikely that spontaneous protected centres are an artifact of the experimental protocol.

Cell lines forming interferon constitutively would be invaluable for investigating the genetics and mechanisms of interferon production and as a source of interferon itself. However, it is not known if the spontaneous, or u.v.-induced, or EMS-induced cells producing interferon represent a random induction of interferon production (perhaps in the course of cell death) or a multiplying subpopulation of constitutively producing cells. The observation that the number of spontaneous centres depends on the condition of the stock plates used to make the assay plates argues that cell death may be involved. However, u.v. or EMS killed cells at a far greater rate than it induced protected centres so that interferon induction is not an invariable consequence of cell death. Nevertheless, it is improbable that a direct mutagenic action of u.v. or EMS is solely responsible, since, at the highest doses investigated, there was approximately one producing cell per 10⁸ surviving cells – an improbably high rate of mutagenesis. Fluctuation tests to settle this point have given equivocal results (unpublished data). In addition, interferon producing cells, whether induced by EMS or u.v. irradiation, were lost during trypsinization and replating, perhaps because of the toxic effects of these treatments or of interferon itself (de Clercq & de Somer, 1975). If interferon-producing cells divide relatively slowly, it will be quite difficult to isolate or even to enrich for them.

We have described a simple and convenient system for the study of interferon production.
by individual cells. The procedures should be applicable to a wide variety of cell/inducer systems. The requirements are few: (1) cells that respond to suitable inducers by producing and releasing interferon, and in the presence of interferon develop an adequate antiviral state; and (2) a challenge virus which does not kill cells that produce only small amounts of interferon.

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