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Effect of Cell Density on Development of the Antiviral State in Interferon-Producing Cells: A Possible Model of *in Vivo* Conditions[1] (39826)

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Protection of the uninfected cell by interferon can proceed rapidly under simulated *in vivo* conditions with treatment by high concentrations of interferon at carefully maintained body temperature (1). Such high concentrations of IF are physiological, as the occurrence of these high concentrations of interferon around interferon-producing cells *in vivo* has been estimated by calculation (1, 2) and subsequently measured experimentally using Millipore diffusion chambers implanted into the subcutaneous tissues of rats (2-4). The effects of these locally high concentrations of interferon on the interferon-producing cell, however, are unknown. The present study utilized differences in interferon concentration around producing cells to help define some of the variables which govern the action of interferon on the same cells which produce the interferon.

Materials and methods. All the experiments were performed using mouse L cells grown either in suspension (for interferon induction) with Eagle's spinner medium or in monolayers with regular Eagle's medium (for interferon assay). The media were supplemented with fetal calf serum (10% for growth and 2% for the experiments) plus antibiotics.

Interferon induction was performed as described under experimental design, using Newcastle disease virus, vaccine strain F (NDV), for induction and encephalomyocarditis virus, mengo strain, for challenge (mengovirus). NDV was propagated in embryonated chicken eggs, mengovirus was propagated in mouse L cells. Aliquots were stored frozen at −70°. NDV was titered as 50% egg infectious doses and mengovirus was titered by plaque assayed in mouse L cells or by hemagglutination.

Interferon titrations were performed in L cells as a single-cycle hemagglutination yield reduction using GD-7 virus as the challenge virus, as previously described (5). Interferon titers are expressed as reference units per milliliter. The assay of the antiviral state was also performed by single-cycle HA yield reduction, but using mengovirus as the challenge virus (6). A threefold reduction with respect to the control virus yield was considered significant.

Results. Plan of the experiments. To help determine the rate and mechanisms of induction of the antiviral state in interferon-producing cells, experiments were designed to vary the concentration of secreted, extracellular interferon and to determine the resulting kinetics of development of the resistant state. The basic manipulation was to vary the extracellular volume around a constant number of interferon-producing cells such that secreted interferon would occur in different extracellular concentrations, but where we would expect no alteration in the amount and concentration of the production of intracellular interferon. Mouse L cells suspended in Eagle's spinner medium were stimulated to produce IF with NDV at an input multiplicity of 50 EID_{50}/cell to simultaneously induce the entire cell population. Virus adsorption was performed at 0°. One hour later unadsorbed virus was removed by three cycles of centrifugation in a refrigerated centrifuge (500*g* for 5 min) and the cells were resuspended at a concentration of 5×10^6 cells/ml in prewarmed Eagle's medium supplemented with 2% fetal calf serum and 0.025 *M* Hepes buffer. One-milliliter samples of the above suspension were

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then distributed either in empty tubes or in centrifuge bottles containing 99 ml of prewarmed Eagle's medium. Consequently, the two kinds of vessels contained the same total number of cells but one had a cell concentration of 5×10^6 and the other had 5×10^4 cells/ml. The cells were incubated in a water bath at 37° with gentle agitation and, at pre-established times, duplicate samples were collected. The fluids were assayed for IF and the cells were washed and challenged with mengovirus to establish the level of antiviral resistance. The mengovirus HA yield was determined after overnight incubation. Comparable sets of cells, run in parallel but not induced with NDV, were used as virus controls.

Effect of cell density on the development of the antiviral state by cells induced with NDV. As specified in the experimental design, cells were induced with NDV and then incubated at high (5×10^6 cells/ml) and low (5×10^4 cells/ml) concentrations during the development of the antiviral state. The results of a representative experiment are shown in Fig. 1. The antiviral state developed much more rapidly in the cells incubated at high density as compared with those incubated at low density. For example, significant resistance developed at 30 min in the high-density cell population and rapidly progressed to complete inhibition of yield of viral HA at 4 hr. In comparison, in the low-density cell population, the antiviral state was first detectable at 2 hr, the rate of development of the resistance was slower, and the yield of viral HA was not completely eliminated.

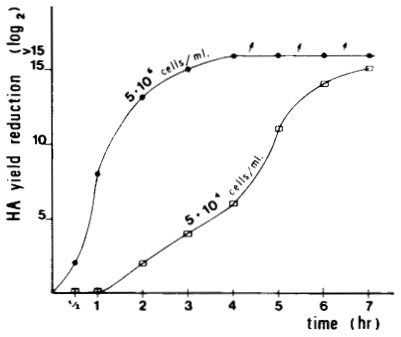

FIG. 1. Development of antiviral resistance to mengovirus in cells induced with NDV (50 ID_{50}/cell) and subsequently resuspended at different cell concentrations. Arrows indicate inhibition of HA yield at the lowest dilution tested.

Effect of cell density on interferon production and concentration in cells induced by NDV. The same cultures which were used to study the development of resistance were used to follow the production of interferon and its concentration at various times after induction by NDV. The results are shown in Table I. At high cell concentration, where the interferon was secreted into a small extracellular volume, the concentration of interferon in the medium surrounding the cells was high (100 to 320 units/ml). In comparison, the cells at low density (the same number of cells suspended in a large volume of medium) produced low concentrations of interferon (2 to 4 units/ml), although the total amount of interferon produced was the same as for the cultures at high density. Thus, the production of interferon per cell was the same under both conditions, but at high density the concentration of interferon around each cell was substantially greater (Table I) and was associated with the development of greater antiviral activity (Fig. 1).

Characterization of the antiviral state induced by NDV in mouse L cells. In the present experiments the development of antiviral resistance was associated with interferon production. Previous studies in the same NDV–mouse L-cell system (1, 7) provided evidence that this antiviral state has

TABLE I. EFFECT OF CONCENTRATION OF MOUSE L CELLS ON PRODUCTION OF INTERFERON AFTER INDUCTION BY NDV.

Cell concentration (cells per milliliter)/total number of cells	Interferon concentration (units per milliliter)/total units of interferon produced at the indicated hour					
	0.5	1	2	3	4	7
$5 \times 10^6/5 \times 10^6$	<10/<10	<10/<10	<10/<10	100/100	320/320	320/320
$5 \times 10^4/5 \times 10^6$	<2/<200	<2/<200	<2/<200	2/200	4/400	4/400

the characteristics of the interferon system. To further establish that the decrease of virus yield in the present experiment was interferon mediated, the requirement for RNA synthesis was studied. Utilizing the experimental conditions employed above the cultures were pretreated with 2 μg/ml of actinomycin D for 1 hr prior to induction by NDV (Table II). Pretreatment with actinomycin D markedly suppressed the development of the antiviral state under conditions of both high and low density, thereby indicating the requirement for RNA synthesis for development of antiviral activity. This property coincides with that of the interferon system. In addition, this finding rules out the possibility that other types of viral interference could have played an important role in the reduction of viral yield.

TABLE II. EFFECT OF ACTINOMYCIN D ON THE DEVELOPMENT OF THE ANTIVIRAL STATE INDUCED BY NDV IN MOUSE L CELLS.

Cell concentration (cells/ml)	Treatment with actinomycin D	Inhibition of mengovirus HA yield ($\log_2$) at the indicated hour after induction by NDV				
		0.5	1	2	3	4
5×10^6	No	2	7	11	13	16
5×10^6	Yes	0	3	4	4	4
5×10^4	No	0	0	2	4	5
5×10^4	Yes	0	0	0	0	2

Discussion. The finding that the rate and degree of development of antiviral activity in cells induced to produce interferon are affected by cell density has several important implications for the mechanisms of action of interferon and its possible role during viral infections *in vivo*. The high concentrations of interferon around densely packed producing cells can be responsible for the resistance to virus challenge applied 30 min after induction of interferon (1). This appearance of resistance to virus challenge at 30 min, as seen in Fig. 1, does not necessarily correspond to the time of the transcription and translation of the antiviral protein(s) because there is a 30- to 45-min interval after virus challenge when newly produced AVP can still block virus replication (7). Thus a conservative estimation of the time required for production of the antiviral protein by the concentrated cells must include the challenge time (30 min) plus the time necessary for derepression of the AVP cistron (30–45 min). It may, therefore, be inferred that the actual production of AVP occurred not later than 60–75 min after induction by NDV. Since interferon synthesis must precede production of AVP (8), it may also be concluded that under the present conditions the time of first production of interferon must have occurred not later than 30–45 min.

The dependence of resistance in the interferon-producing cell on cell density indicates that interferon must be externalized by the producing cell before it acts on the same cell to induce resistance. This conclusion is based on the findings that: (a) The concentration of interferon in the fluid around cells is a major determinant of the degree of antiviral activity (9); (b) in the present study the interferon-producing cells yielded the same total amount of interferon per cell regardless of cell density; and (c) the concentration of secreted interferon in the fluid surrounding the producing cells was inversely proportional to the cell density. Thus, the interferon-producing cells at different densities developed different levels of antiviral activity in relation to the concentration of interferon in the surrounding fluid rather than in relation to the same internal (total) amount of interferon which they produced. This interpretation is consistant with previous studies which indicated that interferon was externalized by the producing cell before it could induce an antiviral effect in the same cell (10, 11).

Although it does appear that interferon must be externalized by the producing cell before inducing antiviral activity in that cell, the degree of resistance which develops is much greater than that which would develop in nonproducing cells exposed to the same concentration of interferon. For example, as shown with the low-cell-density population in Table I, resistance of greater than 32,000-fold inhibition of virus yield was associated with only 4 units/ml of secreted interferon. If these 4 units/ml of interferon were applied to nonproducer cells, the final level of resistance attained would be only 1.5 $\log_{10}$ inhibition of virus yield (9). This finding, taken together with the require-

ment for externalization of interferon for antiviral action in the producing cell (10, 11), implies that the secreted interferon may occur in high concentration at the membrane of the producing cell (before diffusing away into the medium) in order to induce the strong antiviral activity. This possibility, that the site of secretion is close to the membrane receptor for interferon action, merits further study. Such a mechanism could also explain earlier findings that interferon-producing cells develop detectable resistance to virus before they begin to produce detectable levels of interferon (8).

It is not known whether the present findings apply to other cell or virus systems. For example, the inducing virus, NDV, is not cytolytic in the mouse L-cell system and does not inhibit general cell macromolecular synthesis. The kinetics might be expanded to different cell concentrations and viruses which inhibit cell metabolism.

The density of most cell types in the body is even greater than the dense cell population used in the present study. Thus the observed dependency of development of resistance on degree of cell density in the present *in vitro* studies may imply an even greater effect *in vivo*. It is suggested, therefore, that tissue culture models of animal infections utilize highly concentrated cells in order to properly evaluate host defenses during viral infection.

Summary. Tissue culture conditions which mimic the high cell densities *in vivo* were used to elucidate further the virus, cell, and interferon interactions. To help define some of the variables which govern the action of interferon in the same cells which produce the interferon, the technique of altering the effective interferon concentration by varying the extracellular volume was used. Specifically, mouse L cells were treated with a high-multiplicity Newcastle disease virus at 0° and then resuspended at 37° in two different volumes of prewarmed medium such that each had the same total number of cells but the concentration of cells in one culture was 5×10^6 and that in the other was 5×10^4. Under these conditions both cell suspensions produced the same total amount of interferon, but at interferon concentrations of 320 and 4 units/ml, respectively. Antiviral resistance developed more rapidly and to higher levels in the more concentrated cells. It was calculated that at the high cell density (closer to that of solid tissue) the production of the antiviral protein occurred not later than 60–75 min after the induction by NDV and the production of interferon itself must have occurred even earlier. Also, the finding that interferon-producing cells developed different levels of antiviral activity in relation to the interferon concentration in the surrounding fluid, rather than to the same internal (total) amount of interferon produced, supports the view that interferon must be externalized to induce resistance. Finally the possibility was raised that secreted interferon might more efficiently induce resistance in the interferon-producing cell than in nonproducing cells.

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