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Quantitative Aspects of Interferon-Induced Plaque Reduction: Effects of Cell Age and Dose of Challenge Virus (37948)

GEORGE W. JORDAN[1]
(Introduced by T. C. Merigan)
U. S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland

The age of cells cultured *in vitro* has been shown to affect their ability to produce and respond to interferon. Carver and Marcus (1) showed that aged chick embryo cells produced more interferon and were more sensitive to its action when compared with young cells. Lockart (2) found that interferon-induced resistance developed more rapidly in aged chick cells. The importance of both time in culture and cell density was shown by Rossman and Vilcek (3). If the aging process serves only to recruit more cells to a state of interferon sensitivity, less virus would be produced by aged, interferon-treated monolayers but the slope of the dose-response should be unchanged. However, a change in the dose-response would imply that the mechanism of interferon action is directly affected by aging. In the process of studying several factors that affect the degree of plaque reduction induced by interferon, the effect of the *in vitro* age of chick embryo cells and L-cells on the dose-response has been determined. In addition, the relationship of the percentage of plaques to the amount of vesicular stomatitis virus (VSV) used for challenge has been studied for chick and mouse interferon.

Materials and Methods. Cell Cultures. Five milliliters of a 10-day chick embryo cell (CEC) suspension in Medium 199 with 10% fetal bovine serum (FBS) were dispensed into 60-mm plastic petri dishes at a density of 3.5×10^6 cells/ml. After overnight incubation, confluent monolayers were obtained. The cultures were refed Medium 199 with 2% FBS, penicillin (250 μg/ml), and streptomycin (100 μg/ml) (maintenance medium) and were incubated for different periods of time (ages) before use. The cell counts did not differ significantly for the same batch of cells 1 through 8 days after planting.

Five milliliters of an L-cell suspension were dispensed into petri dishes at a density of 3×10^5 cells/ml in Medium 199 with 5% FBS. Confluent monolayers were obtained by the second day when the cultures were refed maintenance medium. Sample monolayers were trypsinized and the cells counted during the aging period.

Experiments on the production of interferon were carried out in 250-ml plastic flasks containing 30 ml of either CEC or L-cell suspension.

Virus strains and interferon. Chick embryo cells were inoculated with the Indiana strain of VSV at low multiplicity and the medium was collected after 18 hr incubation at 37°. The medium was clarified by low-speed centrifugation and had a titer of 1.0×10^9 plaque-forming units (PFU)/ml on CEC. Rio Bravo (RB) virus, a group B arbovirus, strain HA-119, and Newcastle disease virus (NDV) strain B_1 were used to induce interferon in CEC and L-cells respectively (4).

Plaque reduction technique. After a designated period of aging, the cell cultures were refed 3.6 ml of maintenance medium. Samples of each of 5 twofold dilutions of interferon were stored at −20°. When required, a complete set of 5 dilutions was thawed

[1] Present address: Division of Infectious Diseases, Stanford Medical Center, Stanford, California 94305.

and 0.4 ml was added to 3 cultures at each dilution. Interferon remained in contact with CEC for 6 hr and L-cells for 18 hr. The monolayers were then challenged with approximately 100 PFU of VSV and overlaid with maintenance medium containing 0.6% Ionagar. Cells from the same batch were used within each aging experiment; the "cell age" refers to the day after seeding that the monolayers were challenged with virus. Inoculated chick cells were incubated for 20 hr and the L-cells for 40 hr. Neutral red was added 4 hr prior to counting the plaques. The plaque counts at different interferon dilutions were analyzed by the probit method (5, 6).

Results. Effect of cell age on interferon production. In order to show that the cell culture conditions used in these experiments produced the phenomenon of cell aging, the production of interferon in aged CEC and L-cells was determined. The increase in titer of chick interferon induced with Rio Bravo virus (Fig. 1) was progressive over an 11-day period. The increase in titer of mouse interferon (Table I) was abrupt and did not change with further aging of the L-cells. These results indicate that the phenomenon

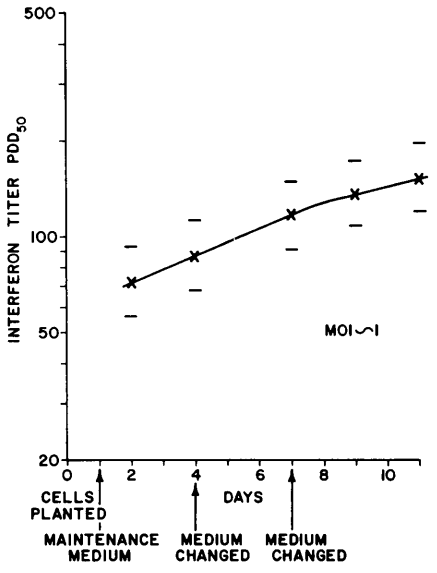

FIG. 1. Effect of cell age on the production of chick interferon by Rio Bravo virus. The 95% confidence intervals are shown.

TABLE I. Effect of Age on the Production of L-cell Interferon by NDV.

Age of monolayers (days)	Interferon titer (PDD_{50})	
3	280	(maintenance medium added)
5	4000	
7	3100	
10	5000	(medium changed)
12	4800	

of aging, as evidenced by increased interferon production, occurred with the medium employed in the following experiments.

Effect of cell age on the dose-response. Cell monolayers, aged for different periods of time, were exposed to identical dilutions of interferon and challenged with approximately 100 PFU of VSV. The probits of the percent of plaques developing were regressed on the logarithm of the interferon dilutions and the results of a representative experiment utilizing chick interferon are shown in Fig. 2A. The slope of the dose-response lines increased progressively with cell age as shown. A test for parallelism (5) was rejected for the three sets of data, indicating that the slopes of the dose-response (response rates for concentration) were significantly different on cells of different ages. No consistent effect of cell age was observed on the interferon titer (PDD_{50}). Using the same dilution of VSV stock, average control plaque counts of 98, 52, and 17 were obtained on days 2, 5, and 8, respectively. The above findings were confirmed in two additional experiments using chick cells.

Similar experiments were done using aged L-cell monolayers. Approximately 100 PFU of VSV were used to challenge each monolayer. The control value, however, did not decrease with the age of L-cells. The response rates for interferon concentration are shown in Fig. 2B. In contrast to chick cells, the slope did not appear to change with cell age. The assumption of parallelism was not rejected, indicating that the response rates for interferon concentration did not vary significantly. The percent of plaques appearing was consistently higher for day 3 but

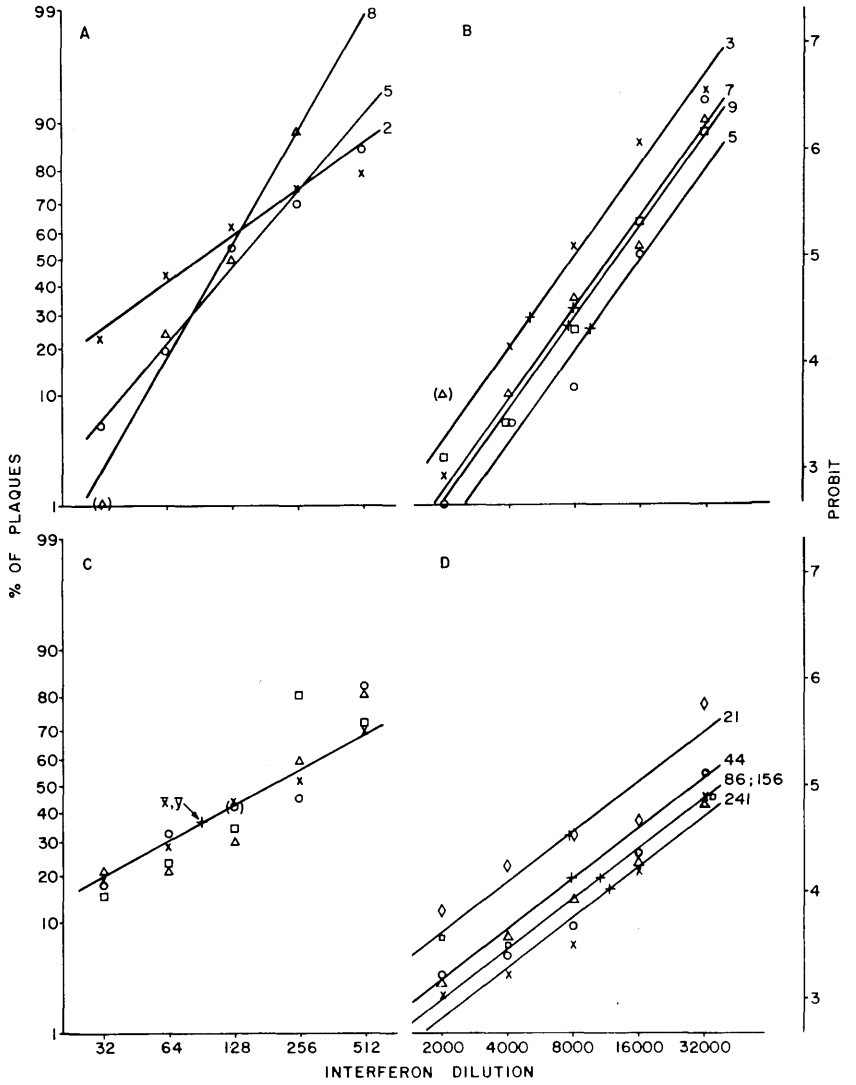

FIG. 2. Interferon-induced reduction of VSV plaques. Symbols represent the average of 3 plaque counts.

(A) Effect of *in vitro* age of CEC. Cell age: (×) 2 days; (○) 5 days; (△) 8 days.

(B) Effect of *in vitro* age of L-cells. Cell age: (×) 3 days; (○) 5 days; (□) 7 days; (△) 9 days. The observation in parenthesis was omitted from the analysis because of nonlinearity.

(C) CEC monolayers challenged with different amounts of VSV. Average of plaque counts on control plates (N): (×) 163; (○) 74; (△) 38; (□) 24. The symbol in parenthesis represents 2 plaque counts.

(D) L-cell monolayers challenged with different amounts of VSV. Average of plaque counts on controls (N): (×) 241; (○) 154; (△) 86; (□) 44; (◇) 21.

systematic deviations were not present for days 5 through 9. Once the cell count of the monolayers reached a constant value, after day 3, the titer of the interferon preparation did not change significantly with further aging of the cells (Fig. 2B).

Effect of the number of PFU for challenge. Cell monolayers were first exposed to

interferon dilutions and then were challenged with different amounts of VSV. The average of plaque counts on the controls (N) was used for calculations with the plaque counts from interferon-treated plates challenged with the corresponding number of PFU. The results for chick interferon are shown in Fig. 2C. Systematic deviations are not present for the different levels of challenge virus and the slopes and titers for the individual lines did not differ significantly. All observations were then used to determine the common line shown in Fig. 2C. The value of chi square (6) for the common line is within the expected range ($\chi^2(57) = 61.7$). Hence, the data are consistent with the assumption that the degree of plaque reduction on CEC is independent of the dose of challenge virus in the range studied.

The percent of plaques appearing on interferon-treated monolayers of L-cells challenged with different amounts of VSV is shown in Fig. 2D. In contrast to the results for chick cells, the L-cell monolayers that were challenged with the fewest PFU consistently gave the greatest percentages of plaques. The assumption of a common dose-response slope was not rejected by the test for parallelism and the comparable interferon titers showed a progressive increase with increasing amounts of challenge virus. The PDD_{50} appears to reach a plateau when the challenge dose is 86 PFU or greater.

Discussion. It was recognized by Isaacs and Baron (7) that tissue from young chick embryos was less sensitive to interferon than tissue from older embryos. Carver and Marcus (1) reported that the aging of chick cell monolayers *in vitro* caused an increase in the titer of an interferon preparation as measured by the plaque reduction method. The results presented here show an effect of *in vitro* chick cell age on the response rate for interferon concentration but a definite effect on the PDD_{50} was not observed. The increased production of interferon and the decreased plaquing efficiency of VSV indicate that the phenomenon of aging did occur in the CEC used in these experiments. The major role of medium composition (1) may be responsible for the smaller magnitude of the aging effects observed here. Similar aging effects were not observed with L-cell monolayers.

The increase in the slope of the dose-response line indicates that for a high concentration of interferon, a greater response will be obtained with aged cells than with young cells. This is consistent with the observations of Lockart (2) and Rossman and Vilcek (3), who showed an increased sensitivity of aged chick cells to the inhibition of virus yield using one or more units of interferon. On the other hand, for lower concentrations of interferon, the difference in response between young and aged cells appears to decrease as implied by the nonparallel dose-response lines and as shown in Fig. 2A. The reason for this finding is not apparent. It might be postulated that aged cells contain fewer interferon receptor sites. This could allow the majority of responses to interferon to occur over a narrower concentration range. In any event, the aging process in chick cells appears to involve the mechanism of interferon action directly and does not merely recruit more cells to a state of sensitivity.

The percentage of plaque inhibition on interferon-treated chick cells using an agar overlay technique was found to be independent of the amount of VSV used for challenge. This is similar to the findings of Lindenmann and Gifford (8) for vaccinia virus plaques formed on chick cells in the presence of interferon. In contrast to chick cells, the percent of plaques appearing on interferon-treated L-cells decreased with increasing amounts of challenge virus. This resulted in an increase in the observed interferon titer as more PFU were used for challenge. The progressive decrease in the percent of plaques obtained suggests the possibility that endogenous interferon, induced by the VSV used for challenge, may add to the effect of the previously applied interferon. In addition, it seems quite likely that the "priming" effect (9) is active here. The shorter time required for the development of VSV plaques in chick cells may not allow endogenous interferon to influence the degree of plaque reduction on these cells.

In the case of L-cells, if the number of challenge PFU is maintained at approximately 100, the influence of minor variations in challenge dose on the degree of plaque reduction should be minimized.

Summary. Increasing the *in vitro* age of chick cell monolayers was found to alter the dose-response to interferon so that aged cells responded over a narrower concentration range than young cells. This effect was not observed on aged L-cells. No consistent effect on the PDD_{50} of chick and mouse interferons was observed under the conditions of aging used in these experiments.

The percent of plaques appearing on interferon-treated chick cell monolayers was found to be independent of the amount of vesicular stomatitis virus used for challenge over an eightfold range. The percent of plaques appearing on interferon-treated L-cells, however, showed a progressive decrease with increasing amounts of challenge virus.

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