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Virus Replication and High-Titered Interferon Production in Human Leukocyte Cultures Inoculated with Newcastle Disease Virus

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ABSTRACT

WHEELOCK, FREDERICK E. (Western Reserve University, Cleveland, Ohio). Virus replication and high-titered interferon production in human leukocyte cultures inoculated with Newcastle disease virus. J. Bacteriol. **92**:1415–1421. 1966.—High titers of interferon (20,480 culture-protecting units per ml) are produced in freshly prepared human leukocyte cultures inoculated with a Newcastle disease virus (NDV)-cell multiplicity of 1:1. NDV replicates to low titers in these cultures. Incubation of leukocytes at 37 C for 24 hr prior to inoculation of NDV results in almost complete loss of detectable interferon production, but virus replicates to higher titers than in the freshly prepared cultures. In contrast, no diminution of interferon production in response to phytohemagglutinin (PHA) occurs on 24 hr of incubation of cultures prior to addition of PHA. Experiments with cultures of predominantly pure cell fractions of peripheral blood indicate that the lymphocyte fraction produces interferon in response to either NDV or PHA, and that polymorphonuclear leukocytes produce no interferon in response to these agents. These studies suggest a hitherto unsuspected ability of human lymphocytes to produce high titers of interferon in vivo.

Interferon production in human leukocyte cultures inoculated with either Sendai virus or measles virus was first reported by Gresser (8). More recently, phytohemagglutinin (PHA), an extract of the kidney bean, *Phaseolus vulgaris*, was shown to induce in human leukocytes an interferon which is acid- and heat-labile (21). In that study, the virus-induced interferon used for comparison with the PHA-induced interferon was made in human leukocyte cultures inoculated with Newcastle disease virus (NDV). Other workers confirmed that interferon is produced in human leukocyte cultures inoculated with myxoviruses (4, 11; H. Strander and K. Cantell, *personal communication*). Strander and Cantell determined the optimal conditions for preparation of leukocyte cultures from large volumes of blood collected in plastic bags and for interferon production in these leukocytes by Sendai virus and NDV.

The present report consists of a further analysis of NDV-human leukocyte interaction and includes the experimental conditions required for production of high-titered human interferon. The inverse relationship between interferon and virus production in leukocytes is described, and, finally, studies on NDV-induced interferon production in predominantly pure cultures of lymphocytes and polymorphonuclear leukocytes are reported.

MATERIALS AND METHODS

Cell cultures. Human diploid fetal lung cells which were pleuropneumonia-like organism (PPLO)-free were grown in Eagle's minimum essential medium supplemented with Tryptose Phosphate Broth (Difco) (4%) and fetal calf serum (10%). The concentration of sodium bicarbonate was 1.75 g/liter, and all cell culture vessels were gassed with 5% CO_2 in air before incubation at 37 C.

The BHK 21 clone 13 (C13), an established cell line derived from baby hamster kidneys (13), was grown in the above medium and was used for assays of NDV infectivity.

In the preparation of leukocyte cultures, the venous blood of healthy adults was placed in tubes containing phenol-free heparin (0.25 ml for each 15 ml of blood). The tubes were incubated at 37 C for 60 min to permit sedimentation of cellular elements; the leukocyte-rich plasma was then removed and centrifuged at room temperature at 250 × *g* for 10 min. The pellet was suspended in growth medium by gentle pipetting.

The leukocytes were counted in a hemacytometer, and the cells were diluted in growth medium to the desired concentration. Screw-cap culture tubes were each planted with 2 ml of the cell suspension, placed in a roller drum, and incubated at 37 C. Each experiment was performed with cells from one donor.

Virus. The Hickman strain of NDV was employed. Virus seeds were grown in the allantoic sac of 11-day-old-chick embryos. The infected allantoic fluids were clarified by centrifugation at 2,500 rev/min for 1 hr and were then centrifuged at 105,000 rev/min for 6 hr. The pellets were washed with phosphate-buffered saline, dispersed sonically, passed through a 450-mμ membrane filter (Millipore Filter Corp., Bedford, Mass.), quick-frozen, and stored at −70 C. The titer of the virus seed employed in these studies was 2×10^9 EID_{50}/ml.

NDV infectivity assay. Infected leukocyte cultures were sonic treated by a Blackstone 400-w ultrasonic probe at maximal power for 15 sec. No loss of NDV infectivity occurred during this procedure. Infective virus was assayed by the tube dilution method in culture tubes containing C-13 monolayers, and the 50% end point was calculated by the method of Reed and Muench (20).

PHA. PHA in 5-ml bottles (Difco) was dissolved in phosphate-buffered saline (100 ml) at room temperature and was passed through a filter with a pore size of 450 mμ. The filtrate was stored at 4 C.

Interferon assay. The specimens to be tested for interferon were diluted in growth medium and 1 ml of each dilution was added to 1-day-old cultures of human fetal lung cells in incomplete monolayers in screw-cap tubes. After 20 hr of incubation at 37 C, the cultures were washed once with 4 ml of phosphate-buffered saline. A 1-ml amount of warm Eagle's medium was added to each tube, and 1 ml of cold growth medium containing 5,000 tissue culture infective doses ($TCID_{50}$) of Sindbis virus (Egypt AR 339 strain) was then inoculated. This amount of virus produces gross cytopathic effects (CPE) in cultures in 24 to 30 hr. Cultures were considered to be protected when there was less than 10% CPE at a time when control cultures exhibited more than 75% CPE.

Interferon titers, expressed as culture-protecting units (CPU), were determined as reciprocals of the highest dilution of the specimen, 1 ml of which protected cultures against challenge with Sindbis virus.

RESULTS

Effect of virus-cell multiplicity on interferon production. The experimental conditions for maximal interferon production by NDV in human leukocyte cultures were first studied. A cell concentration of 2×10^6 cells per milliliter was selected, and the multiplicity of virus inoculated was varied. After virus inoculation, the cultures were incubated in a roller drum at 37 C for 24 hr, an incubation period within which maximal titers of interferon are produced (see below). The leukocytes were then sedimented by centrifugation, the supernatant fractions were collected and acidified at *p*H 2 for 48 hr, then brought to *p*H 7, and the interferon content was assayed as described in Materials and Methods. As will be shown in a subsequent section, acidification at *p*H 2 for 24 to 48 hr inactivates NDV without destroying interferon.

Figure 1 illustrates the effect of multiplicity of virus inoculum on interferon production. Note that maximal interferon production took place in cultures inoculated at a virus/cell multiplicity of 1 EID_{50} per leukocyte.

Effect of leukocyte concentration on interferon production. Human leukocyte cultures were prepared at various cell concentrations and were inoculated with NDV at a virus/cell multiplicity of 1. Cultures were then incubated for 24 hr, and the supernatant fractions were processed for interferon. A direct relationship exists between cell concentrations of $1 \rightarrow 8 \times 10^6$ and the quantity of interferon produced (Fig. 2). Maximal interferon production of 20,480 units per ml was achieved in cultures containing 8×10^6 cells per milliliter; higher concentrations of cells were not studied. No interferon was detected in cultures containing 2.5×10^5 cells.

Temporal relationship between NDV and interferon production. The time course of virus and interferon production in human leukocyte cultures inoculated at a virus/cell multiplicity of 1 was next studied. In these experiments, the leukocytes were washed three times 2 hr after virus inoculation, to reduce the amount of residual unadsorbed virus. The cells were then suspended in growth medium and incubated at 37 C; cultures were collected at intervals thereafter and quick-frozen at −70 C. The cultures were thawed and sonic treated; samples were assayed both for virus infectivity and for interferon as described in Materials and Methods.

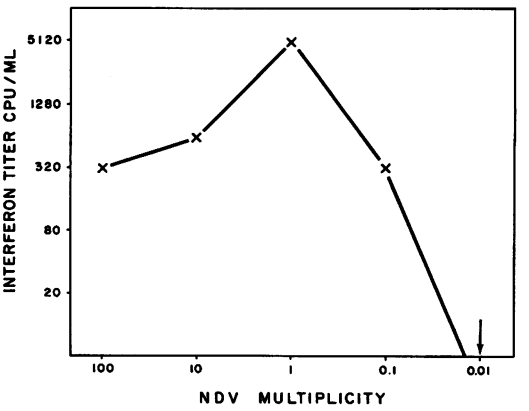

FIG. 1. *Effect of NDV-cell multiplicity on interferon production in human leukocyte cultures.*

Interferon production took place between 2 and 4 hr after virus inoculation; maximal production was reached at 14 hr (Fig. 3). New infective virus was first detected at 7 hr. The persistence of virus titers at the 7-hr level for an additional 65 hr indicates continued replication, inasmuch as thermal inactivation of NDV at 37 C results in over a 2-log reduction in titer in 48 hr. The decline in infective virus between 2 and 4 hr can be explained by the continued eclipse of inoculated virus. Determination of the time of onset of new virus production was difficult in these experiments because of the residual background of virus inoculum. Studies with use of the fluorescent-antibody technique were therefore undertaken. By use of the indirect staining technique with reagents described previously (24), it was found that new virus antigen first appeared in cells at 4 hr after virus inoculation and continued to accumulate until 14 hr. Staining after this time was unsatisfactory in that cellular morphology was grossly distorted, perhaps because of CPE. At a virus/cell multiplicity of 1, only 1 in 100 to 200 cells displayed virus antigen, further explaining the inability to detect early virus production.

It should be pointed out that NDV replication in human leukocyte cultures could not be demonstrated in every experiment performed. The experiment illustrated in Fig. 3 represents one of several in which virus replication was clearly demonstrated.

Effect of incubation of leukocytes in vitro prior to NDV inoculation on interferon production. Human leukocyte cultures were prepared at a concentration of 2×10^6 cells per milliliter and were placed in a roller drum at 37 C. As soon as cultures were prepared, and at intervals thereafter, NDV was inoculated at a virus/cell multiplicity of 1 and incubated for 24 hr; the cultures were processed and assayed for interferon. Figure 4 shows that leukocyte cultures began to lose their capacity to produce interferon within 2 hr after their preparation, and that, by 24 hr, little, if any, interferon was produced in response to NDV inoculation. In contrast to NDV, there was no reduction in interferon production by PHA in leukocytes which were incubated in vitro 24 hr prior to the addition of PHA.

Effect of incubation of leukocytes in vitro prior to NDV inoculation on virus replication. The marked

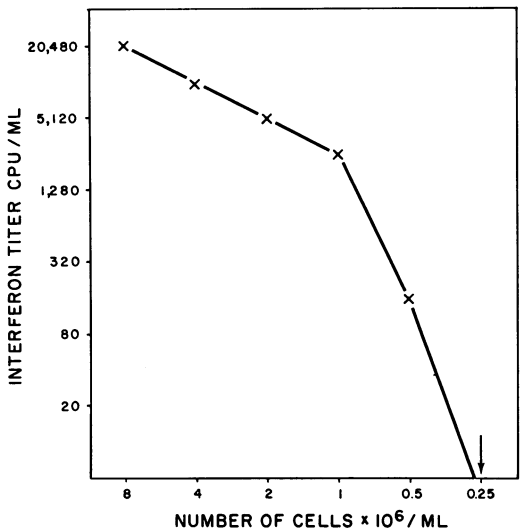

FIG. 2. *Effect of leukocyte concentration on NDV-induced interferon production.*

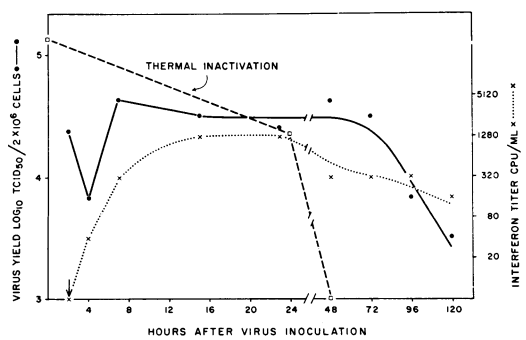

FIG. 3. *Infective virus and interferon production in leukocyte cultures inoculated with NDV. Thermal inactivation of NDV in growth medium was conducted at 37 C.*

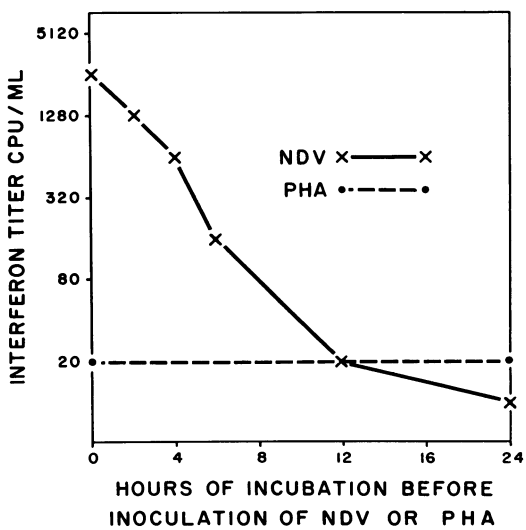

FIG. 4. *Effect of incubation of leukocytes at 37 C before inoculation of either NDV or PHA on interferon production.*

reduction in the interferon-producing capabilities of human leukocytes when incubated in vitro prior to NDV inoculation suggested that virus replication might reach higher titers if NDV were inoculated after impairment of interferon production had occurred. Accordingly, leukocyte cultures were prepared, and immediately, as well as on the 2nd day after preparation, NDV was inoculated into different sets of the culture. Virus and interferon growth curves for both sets of cultures were then determined as described in a preceding section.

Leukocyte cultures inoculated with NDV immediately after preparation produced high titers of interferon, but virus replication was inefficient, with a maximal titer of $10^{4.7}$ reached at 12 hr (Fig. 5). In contrast, 2-day-old cultures produced no detectable interferon, but virus replicated to higher titers, reaching a maximum of $10^{5.8}$ at 12 hr. This experiment demonstrates an inverse relationship between interferon and virus production, and also provides evidence that 2-day-old leukocyte cultures contain viable cells.

Interferon production by lymphocyte and polymorphonuclear leukocyte cultures. To investigate the mechanisms of impairment of virus-induced interferon production, a study of the cell type which was producing interferon in response to NDV inoculation was undertaken. Previous studies by Lee and Ozere (11) showed that human mononuclear leukocytes produce interferon in vitro in response to Sendai virus inoculation, and that polymorphonuclear leukocytes also participate in interferon production.

Human lymphocytes and polymorphonuclear leukocytes were separated in a column of siliconized glass beads by the method of Rabinowitz (19). Lymphocyte cultures were 99% homogeneous; polymorphonuclear leukocyte cultures were 95% homogeneous. The cells of each type were diluted to a concentration of 2.8×10^6 per milliliter, and NDV was added immediately to one sample of each of the two cultures and to other samples at intervals of 8 and 24 hr thereafter. Twenty-four hours after each culture had been inoculated with virus, the cells were removed by centrifugation, and the supernatant fractions were processed and assayed for interferon. The results (Fig. 6) indicate that lymphocyte, but not polymorphonuclear leukocyte, cultures produced interferon in response to inoculation with NDV. Furthermore, within 8 hr after lymphocytes had been placed in culture, they underwent a reduction in their interferon-producing capabilities. Dye exclusion studies with erythrosin B (18) demonstrated that this reduction in interferon production was not due either to destruction of cells or to loss of viability over the time period studied. No significant degree of virus replication could be demonstrated in two experiments with pure cell cultures; further studies with pure and mixed cultures are in progress.

In other experiments with cultures of predominantly pure cell fractions of peripheral blood, it was shown that PHA also induced interferon production in lymphocyte but not in polymorphonuclear leukocyte cultures, and that no reduction in PHA-induced interferon production resulted on incubation of lymphocytes in vitro prior to addition of PHA. These experiments indicate a probable difference between mechanisms of NDV- and PHA-induced interferon synthesis.

Characteristics of NDV-induced human interferon. Some of the biological and physicochemical characteristics of interferon induced in human leukocytes by NDV have been described in a previous publication (21). In brief, this interferon is stable at *p*H 2 and 56 C, is inactivated by tryp-

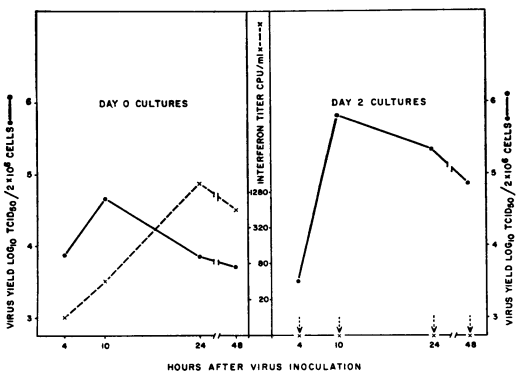

FIG. 5. *Effect of incubation of leukocytes at 37 C before incubation of NDV on virus and interferon production.*

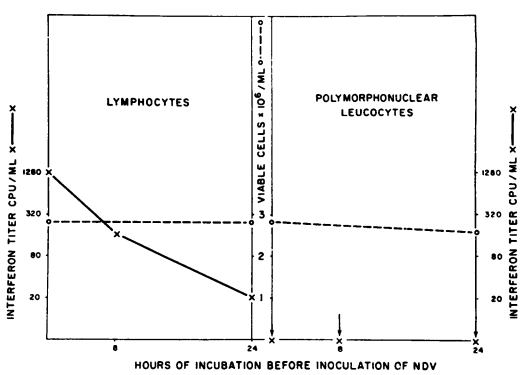

FIG. 6. *Interferon production in lymphocyte and in polymorphonuclear leukocyte cultures inoculated with NDV.*

sin, but not by nucleases, is not sedimented at 105,000 × *g* for 3 hr, and protects human, but not mouse or hamster, kidney cell monolayers against virus-produced CPE. More recent studies in conjunction with T. C. Merigan (*unpublished data*) indicate that the NDV-induced interferon has a molecular weight of 19,000.

The infective virus present after a 24-hr incubation of leukocyte cultures inoculated with a virus/cell multiplicity of 1 could be completely inactivated at *p*H 2 for 24 hr. Higher multiplicities of virus inoculum required 48 hr of acidification for complete inactivation. In these experiments, the culture supernatant fraction was acidified at *p*H 2 for 24 and 48 hr and then assayed for virus and interferon; the titers were compared with another sample of the same supernatant fraction which had been mixed with anti-NDV immune serum at a titer sufficient to neutralize all virus. No reduction of interferon titer resulted from the acidification procedure over the time periods studied.

Effect of PHA on NDV-induced interferon production. The ability of PHA to induce interferon in human leukocytes raised the question as to whether PHA would enhance NDV-induced interferon production in leukocyte cultures.

Human leukocyte cultures in 2-ml volumes were prepared at a cell concentration of 2 × 10^6 per milliliter and were inoculated with 0.1 ml of 1:100 dilutions of PHA. In separate cultures, NDV was added alone, together with, or at intervals after, PHA, and the supernatant fractions were harvested 24 hr after the last agent was inoculated. The results shown in Table 1 indicate that PHA does not enhance, and, indeed, may slightly inhibit, NDV-induced interferon production.

TABLE 1. *Effect of PHA on NDV-induced interferon production in human leukocyte cultures*

PHA on day[a]	NDV on day[a]	Interferon titer (CPU/ml)
—	0	10,240
—	1	40
—	2	20
0	0	2,560
0	1	10
0	2	10
0	—	40
2	—	40
—	—	0

[a] Leukocyte cultures were prepared and incubated at 37 C for the number of days indicated prior to inoculation of PHA or NDV. Cultures were harvested 24 hr after the last agent was inoculated.

DISCUSSION

Human leukocyte cultures inoculated with NDV constitute a rich and readily available source of human interferon. Highest titers of interferon are produced in freshly cultured leukocytes inoculated with a virus/cell multiplicity of 1. There are several nonhuman cell systems described in the literature (3, 5, 12) in which the highest titers of interferon were produced in cultures inoculated with a virus/cell multiplicity less than the maximum employed. The present report is in agreement with these previous studies.

Of unusual interest in the experiments presented here is the marked reduction in the interferon-producing capabilities of human leukocytes when they are incubated in vitro prior to virus inoculation. Strander and Cantell (*personal communication*) found that human leukocytes incubated for 24 hr prior to virus inoculation produced one-third as much interferon as did freshly cultured leukocytes. The more marked reduction in interferon production reported in the present studies may be related to the higher titers of interferon produced in leukocytes exposed to NDV immediately after preparation of cultures: 5,120 units as compared with 300 units in Strander and Cantell's studies. Another possible explanation for this quantitative (but not qualitative) difference could be the techniques employed in the preparation of leukocyte cultures.

The studies with predominantly pure cultures of lymphocytes and polymorphonuclear leukocytes indicate that the lymphocyte fraction of peripheral white blood cells is a source of NDV-induced interferon production. Other types of mononucleated leukocytes, not studied in these experiments, may also contribute to interferon production. It should be noted that there was no loss of viable cells in the first 24 hr of lymphocyte culture, during which time the interferon-producing capability of the culture was reduced by 2 logs. This fact suggests that reduction in interferon production is not due to loss of cell numbers during incubation, but is probably the result of biochemical alterations which take place shortly after transfer of cells from in vivo to in vitro systems. It should be noted that these alterations affect virus-induced interferon but not PHA-induced interferon production: freshly cultured leukocytes produced as much interferon in response to treatment with PHA as did 3-day-old cultures.

The data presented indicate that replication of NDV in human leukocytes is inversely related to the interferon-producing capabilities of the cells. The ability of viruses to replicate to higher titers in cultures in which interferon production has

been suppressed by actinomycin D (10) or by other viruses (14) has been previously reported. A variety of viruses has been reported to replicate in human leukocyte cultures. These include measles virus (2), echovirus 9 (1), poliomyelitis virus (9), herpes simplex virus (16), rubella virus (15), vesicular stomatitis virus (R. Edelman and E. F. Wheelock, Bacteriol. Proc., p. 134, 1966), and mumps virus (4). It is of interest in these studies that, with the exception of poliomyelitis virus, all experiments were conducted in freshly prepared cultures, and low yields of virus production were observed. In contrast, Gresser inoculated cultures with poliomyelitis virus immediately after preparation and observed a 5-day eclipse period, whereas cultures inoculated 4 days after preparation produced new virus in only 2 days. Both sets of cultures produced high titers of virus, but no comparison was made of the maximal final titers. Although no interferon studies were conducted, it is possible that the shortened poliomyelitis virus eclipse period in 4-day-old human leukocyte cultures as compared with freshly prepared cultures might have been due to an impairment of interferon production similar to that reported here.

It is probably incorrect to apply the results obtained in these leukocyte experiments to other human cell types. In contrast to most other cells, peripheral leukocytes are end-stage forms which have no mitotic potential except when stimulated by certain mitogenic agents such as PHA. In support of the hypothesis that human leukocytes are different from other cell types in their interferon responses to virus infection is the finding that chick embryo fibroblasts on prolonged culture in vitro produce increasing amounts of interferon in response to virus inoculation (D. H. Carver and P. I. Marcus, Bacteriol. Proc., p. 124, 1966).

Finally, the markedly reduced interferon-producing capacity of human leukocytes when transferred from in vivo to in vitro suggests that evaluation of the importance of interferon as a mechanism of host defense against viral infections should be, and perhaps can only be, conducted in vivo. In this respect, the detection in man of circulating interferon after vaccination with the active 17 D strain of yellow fever virus (23), measles virus (17), influenza virus (R. L. Jao, E. F. Wheelock, and G. G. Jackson, J. Clin. Invest., p. 1062, 1965), and a variety of clinical viral infections (22) may reflect a hitherto unsuspected ability of human leukocytes and perhaps other cell types to produce high titers of interferon in vivo. The demonstrated ability of mouse leukocytes to produce interferon in response to vaccinia (6, 7) and Sendai virus and NDV (L. Borecky and V. Lackovic, *personal communication*) may provide experimental systems to study further the in vivo role of leukocytes in viral infections.

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