PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 164, 146–152 (1980)

Rapid Onset of the Interferon-Induced Antiviral State in Human Nasal Epithelial and Foreskin Fibroblast Cells (40838)

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Interferon (IF), by virtue of its broad spectrum of antiviral activity and low toxicity, would be an ideal agent for use against viral respiratory tract infections. However, clinical trials have indicated an IF dose requirement larger than would be predicted from the *in vitro* IF sensitivity of the challenge virus in fibroblast cell culture (1). Several hypotheses can be advanced to account for the apparently large *in vivo* dose requirement: (a) inactivation of IF by nasal secretions, (b) rapid clearance from the nasal passages, (c) low overall sensitivity of nasal epithelial (NE) cells to IF, and/or (d) slow rate of activation of the antiviral state in NE cells by IF. Our previously published results indicate that leukocyte IF is not inactivated by nasal secretions (2). We have also shown that IF is rapidly removed from the nasal passages (3) and could partially account for the large *in vivo* dose requirement. The latter two possibilities, relative sensitivity to IF and rate of activation of the antiviral state in NE cells have not been investigated adequately. This report compares the magnitude and rate of activation of the antiviral state in NE and human foreskin Fibroblast (HFF) cells following exposure to IF.

Materials and methods. IF preparation. Human leukocyte and fibroblast IF were provided by the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland. Leukocyte IF was prepared from peripheral blood leukocytes stimulated with Sendai virus and partially purified by Cantell *et al.* (4). Fibroblast IF was prepared by Havell and Vilĉek (5) using human foreskin fibroblasts and polyriboinosinic:polyribocytidylic acid as the inducer. Nasal mucosal IF was obtained from pooled nasal wash specimens in veal infusion broth obtained from volunteers shedding influenza virus after an artificial challenge. Virus was inactivated by treatment at pH 2.0 for 24 hr at 4°C. Following neutralization with NaOH, nasal wash pools were concentrated 10-fold by placing them in dialysis bags and surrounding the bags with Sephadex (G-200). Similar nasal wash specimens from uninfected volunteers were treated in the same manner and served as "mock" nasal mucosal IF. The IF concentrations were 120 units and <2 units/ml for nasal mucosal and "mock" nasal mucosal IF, respectively.

Viruses. The preparation and assay of vesicular stomatitis virus (VSV) have been previously described (6). The HA1 strain of parainfluenza virus type 3 (P-3) was originally obtained from R. M. Chanock. Virus was grown in HeLa-S3 cells (from D. DeLong) and titered $10^{8.1}$ plaque-forming units (pfu) per millimeter in the same cells. The plaque assay for P-3 was described by Glezen and Fernald (7). Coxsackievirus A type 21 (Cox A-21) was an isolate which had been passaged twice in human embryonic fibroblast cells (strain WI-26). The virus was also passaged twice in NE cells and three times in human embryonic fibroblast cells (strain WI-38). This virus was assayed by cytopathic effect production in WI-38 cells and titered $10^{7.1}$ 50% tissue culture infectious doses ($TCID_{50}$) per milliliter. Titers of Cox A-21 were calculated by the Kärber method (8).

IF assay. Details of the yield reduction IF assay using HFF cells and VSV have been described (6). Virus yields were determined by plaque assay in L cells. A laboratory IF standard was included in each assay and the titer never varied more than twofold. One unit of IF was defined as the dilution of the original sample that reduced the VSV yield by 50% when compared with controls. A unit of IF was equivalent to 0.4 unit of human IF research standard B

0037-9727/80/060146-07$01.00/0
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(69/19) from the Medical Research Council, Mill Hill, London, England. All IF values are expressed in human IF standard B (69/19) units.

Cell culture. HFF cells (strain HR-218) from HEM Research, Inc. (Rockville, Md.) were used from passage levels seven through 12 for IF assays. Both HFF and mouse L cells were maintained in Eagle's minimum essential medium with Earles' salts (MEM) purchased from GIBCO (Grand Island, N.Y.). MEM was supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

NE cell culture. After obtaining written informed consent, NE cells were removed with a Freimuth currette from the middle turbinates of healthy volunteers (9, 10) (6 to 13 volunteers per experiment). NE cells were placed in a plastic petri dish (60 × 15 mm) and 5.0 ml of medium 199 was added. Medium 199 was supplemented with 2.0% fetal bovine serum, 0.3% bovine serum albumin, L-glutamine (10 m*M*), and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml; amphotericin B, 2.5 μg/ml). Medium 199 was buffered with 0.15% $NaHCO_3$ and 25 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid (GIBCO). Following incubation at 37°C for 30 min, cells were transferred into 15 ml conical polystyrene centrifuge tubes (Falcon Plastics) and centrifuged at 200 *g* for 10 min. Medium was removed, and the cells were dispersed by incubation at 37°C for 10 to 20 min in medium 199 containing 2.0% *N*-acetyl-1-cysteine and 0.05% EDTA. The pH was adjusted to 8.1 for this treatment (11). Experiments with HFF cells indicated treatment with *N*-acetyl-1-cysteine and EDTA did not alter the IF response or virus replication. After two washes in fresh medium 199, cells were counted and viability was estimated by exclusion of 0.04% trypan blue. The pooled cells were aliquoted into clean conical polystyrene tubes (1.5 × 10^5 viable cells/tube) and incubated at 34°C with medium 199 (control cells) or medium 199 containing IF (IF-treated cells). Each experiment was conducted with two to four replicates of control cells and two to four replicates of cells treated with each IF concentration. After IF treatment, cells were washed and challenged with 0.1 ml of VSV ($10^{6.1}$ pfu). Following virus adsorption (37°C, 2 hr) cells were washed five times (2.0 ml/wash) with fresh medium and half of the last wash was removed for residual virus titer determinations. Cells were incubated in the remaining 1.0 ml medium for 24 hr at 34°C. At that time, the 1.0 ml of medium was removed for virus yield determination and the cells were refed with fresh medium 199. This was repeated at 48 and 72 hr after infection. Virus yields were determined by titration in L cells and the results are expressed as the difference ($\log_{10}$) in yields between control and IF-treated cultures. Except where indicated, the virus yield in each culture is the sum of the 24-, 48-, and 72-hr yields minus the residual 0-hr virus titer.

Results. Time and dose–response to IF. Replication of VSV in HFF cells was complete within 24 hr after challenge (i.e., control monolayers were completely destroyed), whereas following a similar virus challenge of NE cells, virus replication continued for a 72-hr period. Thus, to compare the IF response of these two cell types, a common time after challenge had to be selected to determine virus yields. Table I compares the reduction in virus yields from IF-treated NE cells at 24 hr after challenge with the yield reduction observed over the entire 72-hr period. The mean and standard error of control virus yields at 24 hr for the 10 experiments was 5.39 ± 0.16 pfu/ml ($\log_{10}$). Good agreement exists between the 24-hr data and the sum of the 24-, 48-, and 72-hr data. The largest difference between mean values was 0.26 $\log_{10}$.

The demonstration that the total IF response of NE cells was reflected by the 24-hr data allowed for a comparison of the IF response of NE and HFF cells. HFF cells were grown in 16 × 120-mm test tubes to a concentration of 1.5 × 10^5 cells/tube. Cells were treated with leukocyte IF in medium 199 and challenged with VSV as described for NE cell culture. The reductions in virus yields are presented in Fig. 1 along with the 24-hr response of NE cells

TABLE I. TIME AND DOSE–RESPONSE OF HUMAN LEUKOCYTE INTERFERON IN NASAL EPITHELIAL CELLS

IF (units/ml)	Time of IF exposure	Reduction in VSV yield (log_{10}) 24 hr after challenge	24, 48, 72 hr ($\sum$) after challenge
40,000	15 min	1.00 ± 0.03[a]	0.94 ± 0.22
	1 hr	1.30 ± 0.04	1.45 ± 0.04
	18 hr	1.64 ± 0.33	1.87 ± 0.05
4,000	15 min	0.68 ± 0.27	0.43 ± 0.10
	1 hr	1.15 ± 0.13	0.89 ± 0.17
	18 hr	1.53 ± 0.26	1.40 ± 0.06
400	1 hr	0.21 ± 0.49	0.11 ± 0.27
	18 hr	1.11 ± 0.31	1.36 ± 0.36
40	18 hr	0.76 ± 0.19	0.77 ± 0.13
4	18 hr	0.40 ± 0.23	0.39 ± 0.19

[a] Each number (reduction in VSV, log 10) represents the mean and standard error of two or three experiments. Each experiment had two to four replicates at each IF concentration.

(shown in Table I). The control virus yield for HFF cells was 8.16 ± 0.23 pfu/ml (log_{10}). Both types of cells respond to IF in a time- and dose-dependent manner. It should be noted that the antiviral state develops rapidly (15-min exposure) in NE cells provided high IF concentrations are used for treatment. Correspondingly rapid onset of antiviral activity was also observed

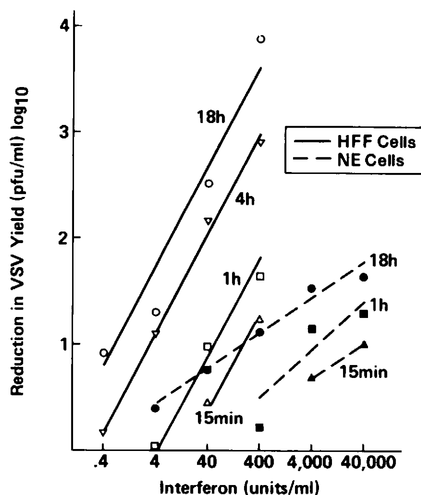

FIG. 1. Comparison of the time and dose–response of interferon in human foreskin fibroblast and nasal epithelial cells challenged with vesicular stomatitis virus. Times indicated in figure represent the time cells were exposed to interferon at 34°C. Virus yields were measured 24 hr after challenge.

when similar concentrations of fibroblast IF were used for treatment (data not shown). The reduction in VSV yields in NE cells following IF treatment was less compared to the reduction observed in HFF cells. In addition, the slopes of the dose–response curves are different, 0.9 and 0.3 for HFF cells and NE cells, respectively.

The observed reductions in virus yields do not appear to be due to a toxic effect of the IF preparation. NE cells treated with 40,000 units of IF for 18 hr showed the same viability as control cells incubated with medium alone. Viability was estimated by trypan blue exclusion.

The VSV yield reductions observed in NE cells 48 and 72 hr after challenge are shown in Fig. 2. Under conditions of VSV challenge, the antiviral state persists for at least 72 hr after challenge. Also, the slope of the IF dose–response of these cells remains 0.3 at 48 and 72 hr after challenge.

Sensitivity of different viruses to IF. Another factor which may influence the effectiveness of IF treatment is the sensitivity of the challenge virus to the IF-induced antiviral state. The sensitivity of two respiratory viruses (Cox A-21 and P-3) to the antiviral state in NE cells is shown in Table II. The response of VSV (from Table I) is shown for comparison. The sensitivity of Cox A-21 appears to be nearly equivalent to that of VSV. Aliquots of the same cell prepara-

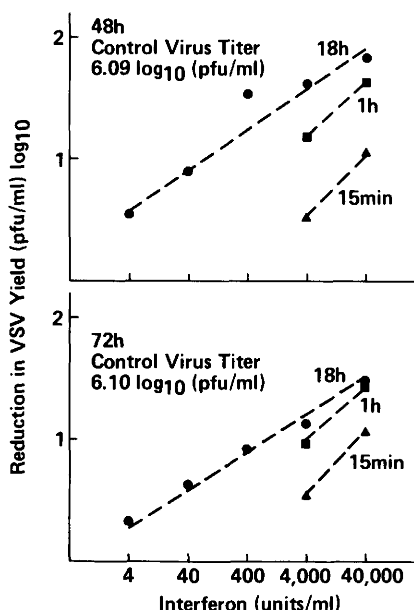

FIG. 2. Time and dose–response of interferon in nasal epithelial cells 48 and 72 hr after challenge with vesicular stomatitis virus.

tions (three experiments) were used for the P-3 and comparable VSV data points. Analysis of variance indicated that the response of VSV to 400 units was significantly greater than that of P-3 ($P < 0.005$).

Effectiveness of IF from different sources. We have shown that interferons from two types of tissue, i.e., leukocytes and fibroblasts, were equally effective in NE cells (6). However, in view of the recent report suggesting tissue specific inhibition of cell growth by IF (12), we tested IF obtained from nasal washes (presumably NE cell IF) in NE cells (Table III). On a unit basis, interferons from two different sources (leukocytes and NE cells) are equally effective in NE cells. Cells treated with "mock" nasal mucosal IF showed no difference in VSV replication compared to NE cells treated with medium 199.

Discussion. Plans for the use of an antiviral agent in man would ideally be supported by an understanding of the action of the agent in the proposed target tissue. In the case of IF, it is well documented that different cells as well as viruses vary in their response to IF (13–16). We have developed and used a human NE cell culture system to verify that these cells respond to IF by becoming antiviral (6, 17). In the present report we describe an improved system in which cells for each experiment are pooled, dispersed, and counted. This provides identical replicates so that data on multiple IF concentrations can be gathered on the same cells with a minimum of variation. Using this system it was shown that both NE and HFF cells respond to IF by development of an antiviral state that is influenced by the length of exposure to, and the

TABLE II. SENSITIVITY OF DIFFERENT VIRUSES TO THE HUMAN LEUKOCYTE INTERFERON-INDUCED ANTIVIRAL STATE IN NASAL EPITHELIAL CELLS

IF (units/ml)	Time of IF exposure (hr)	Reduction in virus yield ($\log_{10}$)		
		VSV	Cox A-21	P-3
40,000	1	1.45 ± 0.04[a]	0.72 ± 0.09	—[b]
	18	1.87 ± 0.05	1.24[c]	—
4,000	1	0.89 ± 0.17	0.41 ± 0.09	—
	18	1.40 ± 0.06	0.73 ± 0.24	—
400	18	1.36 ± 0.36[d]	0.78 ± 0.27	0.55 ± 0.18[d]
40	18	0.77 ± 0.13[d]	0.83 ± 0.15	0.44 ± 0.08[d]
4	18	0.39 ± 0.19[d]	0.32 ± 0.24	0.29 ± 0.08[d]

[a] Each number (reduction in virus yield, log 10) represents the mean and standard error of two to four experiments. Each experiment had two to four replicates at each IF concentration.
[b] Not tested.
[c] Experiment not repeated. Reduction in Cox A-21 yield represents the mean of three replicates of NE cells.
[d] Tested in replicates of the same population of NE cells.

TABLE III. COMPARISON OF HUMAN LEUKOCYTE AND NASAL MUCOSAL INTERFERONS IN NASAL EPITHELIAL CELLS

IF source	(units/ml)[a]	Reduction in VSV yield ($\log_{10}$)	
Leukocytes	120	0.73[b]	
Nasal mucosa	120	0.93[b]	
Nasal mucosa	120	0.58	(0.76 ± 0.18)[c]

[a] IF exposed to NE cells for 4 hr at 34°C.
[b] Tested in replicates of the same population of NE cells.
[c] Mean reduction (± standard error) of VSV yield in NE cells exposed to IF from the nasal mucosa.

concentration of IF. However, the magnitude of the antiviral state and the slopes of the dose–response curves were markedly different for the two cell types. The explanation for the difference in slopes of the dose–response curves for the two cell types is uncertain. It may be due to differences in the number or avidity of IF receptors or to differences in any number of cellular events leading to development of the antiviral state. Although the diminished response of NE cells to IF suggests that this might play a role in the apparently high *in vivo* dose requirement, this cannot be stated with certainty until the level of the antiviral state in NE cells, as measured in this system, is correlated with clinical response.

It has been clearly shown by others that different viruses exhibit different sensitivities to IF-induced resistance in the same cell when measured by single and multiple replication cycle assays (16, 18, 19). Of particular interest is the data of Came *et al.* (20) who demonstrated that a number of rhinovirus serotypes were equally sensitive to IF when tested in fibroblast cells but showed markedly different sensitivities to IF when measured in HeLa cells, a heteroploid epithelial cell line. We have shown here that Cox A-21 and P-3 are slightly less sensitive to the IF-induced antiviral state in NE cells compared to VSV. These differences occurred only at the highest IF concentration tested in each case. Although different challenge virus inocula were used, Hallum and Youngner have demonstrated that these differences in challenge inocula should not affect the IF sensitivity results (21). If these differences in IF sensitivity are clinically significant, they suggest that *in vitro* prediction of clinical effectiveness of IF against respiratory virus disease would require measurement of the IF sensitivity of the challenge virus in NE cells.

Since virus yields in NE cells were measured 24, 48, and 72 hr after challenge, we could determine the level of antiviral activity at each of these time points. It is interesting to note that under conditions of virus challenge the antiviral state appears to persist for at least 72 hr following removal of IF. The maximal reduction in virus yields were observed in the 72-hr samples for P-3 and Cox A-21, while in NE cells challenged with VSV, the peak reduction occurred 48 hr after challenge. It has been shown that cycloheximide will prevent the decay of the antiviral state following removal of IF, presumably by blocking the synthesis of a regulatory protein (22). The viruses themselves may be responsible for a similar effect since most viruses reduce cellular protein synthesis to some extent. Additionally, in our experiments cells were challenged with virus immediately after removal of IF. Hallum *et al.* (13) have shown that in the same cell the antiviral state is lost at different rates for different viruses. Thus, if we had delayed challenge of these cells, we may not have observed a prolonged antiviral state in all cases.

Another factor to be considered for the successful clinical application of IF is the delivery system. We have previously shown that the method of delivery of IF to the nasal mucosa greatly influences the results (17). Significant antiviral activity following *in vivo* IF application was found when IF was applied by a saturated cotton pledget (independent of antihistamine pre-

treatment of volunteers) or by drops in volunteers pretreated with antihistamines. Application of IF by drops in volunteers not pretreated with antihistamines was unsuccessful. These results strongly suggest that clearance and/or access of IF to NE cells is a major obstacle in the treatment of the nasal passages with IF.

Since it is unlikely that we will be able to overcome the obstacle of access to nasal cells for prolonged periods of time *in vivo*, an important aspect of these studies is the demonstration that NE cells develop significant antiviral activity after a brief *in vitro* exposure to IF. Dianzani and Baron (23) were the first to demonstrate the rapid onset of antiviral activity. A 1- to 30-min exposure to IF resulted in a high, but not maximal, antiviral response; 2–4 hr were required for maximal titers to develop. The responses were proportional to the IF concentration. In the present report the conditions necessary to achieve a 10-fold decrease in virus replication in NE cells were treatment with 400 units for 18 hr, 4000 units for 1 hr, or 40,000 units for 15 min. Thus, a 100-fold increase in IF concentration reduced the time of contact requirement considerably (72-fold). It is of interest to note that the rapid onset of antiviral activity in NE cells occurred at 34°C which is the temperature of these cells *in vivo*.

Ultimately, it would be desirable to be able to predict clinical efficacy of IF or IF inducers without conducting clinical trials. Such factors as the sensitivity of NE cells to IF, sensitivity of the challenge virus to the antiviral state in NE cells, and, as shown previously, the optimal delivery system, must all be considered. As these results suggest, data from HFF cell cultures may not be totally predictive. Thus, determination of the level of the antiviral state in cells removed from the nasal mucosa following IF application and correlation of that level with clinical success will be required before *in vitro* predictions are feasible.

Summary. This report describes a cell culture system with nasal epithelial (NE) cells in which the interferon (IF) response of these cells was compared with the IF response of human foreskin fibroblast (HFF) cells. The antiviral state that developed in NE cells after exposure to human leukocyte IF was less than the antiviral state that developed in HFF cells. In addition, the slope of the dose–response curve for HFF cells was greater than that for NE cells. It could be demonstrated that the antiviral state developed rapidly in NE cells following a brief (15-min) exposure to IF at 34°C provided a high IF concentration was used. Two respiratory viruses were tested for sensitivity to the IF-induced antiviral state that develops in NE cells. Both coxsackievirus A type 21 and parainfluenza virus type 3 were slightly less sensitive to the IF-induced antiviral state in NE cells compared to vesicular stomatitis virus.

We sincerely appreciate the excellent technical assistance of Michéle Pelanne.

This work was supported by Public Health Service Contracts AI 42530 and AI 32506 from the Development and Applications Branch, National Institute of Allergy and Infectious Diseases.

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Received January 10, 1980. P.S.E.B.M. 1980, Vol. 164.