

Three Strains of Influenza A Virus (H3N2): Interferon Sensitivity in Vitro and Interferon Production in Volunteers

DOUGLAS D. RICHMAN, BRIAN R. MURPHY,* SAMUEL BARON, AND CAROL UHLENDORF

Laboratories of Infectious Diseases and Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014*

Received for publication 17 November 1975

Three antigenic variants of the H3N2 subtype of wild-type influenza A virus (representing the years 1968, 1972, and 1974) were examined for their sensitivity to interferon and for their ability to induce local respiratory tract interferon in volunteers. In addition, the time of appearance of symptoms in infected volunteers was correlated with the patterns of virus shedding and interferon production. The sensitivity to interferon and the ability to stimulate nasopharyngeal interferon were similarly high for all three strains. Symptomatic illness, peak virus shedding, and peak interferon response all occurred within a 26-h period. These findings imply that interferon or its inducers theoretically could be protective if applied prophylactically, but would be less efficacious when used therapeutically.

Volunteer recipients of an early antigenic variant of the H3N2 subtype of wild-type influenza A virus (A/Bethesda/1015/1968) produced moderate to high levels of interferon in the nasopharynx (7). In addition, this strain was shown to be relatively interferon sensitive (7). Interferon has been postulated as a significant factor in the recovery from viral infection and has been considered to have some potential benefit in the chemoprophylaxis and chemotherapy of many viral infections (2, 5). The present study was undertaken to determine if the high level of sensitivity to interferon and the ability to induce nasopharyngeal interferon were general properties of subsequent influenza A viruses within the H3N2 subtype. Two additional low passage strains of influenza A virus, influenza A/Udorn/307/1972 and influenza A/Georgia/101/1974, were administered to seronegative adult volunteers. The kinetics of development of illness, quantitative virus shedding, and interferon response were examined.

MATERIALS AND METHODS

Viruses. The influenza A/Bethesda/1968 (H3N2) virus was isolated in primary human embryonic kidney (HEK) tissue culture and passaged one additional time in HEK tissue culture. Influenza A/Udorn/307/72 (H3N2) was isolated and passaged five times in primary bovine kidney (BK) tissue culture including three plaque-to-plaque purifications before a final passage in the allantoic cavity of specific pathogen-free hen's eggs (Spafas, Storrs, Conn.). The influenza A/Georgia/101/74 (H3N2) was isolated and passaged four times in BK tissue culture including two plaque-to-plaque purifications

before a final passage in eggs. In each case $10^{4.0}$ to $10^{4.5}$ 50% tissue culture infectious doses of the indicated virus was administered intranasally by drops or by DeVilbiss no. 15 coarse droplet atomizer. All influenza viruses were found free of adventitious agents before use (6, 8). The Sindbis virus pool was prepared in chicken embryo cell cultures.

Volunteer studies. Prisoner volunteers received the A/Bethesda/68 wild-type virus and free living volunteers received the two subsequent viruses. For all three studies volunteers were kept in isolation for 2 days before and for 10 days after virus administration to prevent the introduction of extraneous agents and the spread of wild-type viruses to the community. The wild-type viruses were administered as a part of a more extensive program to develop a live influenza A virus vaccine. In this context, the wild-type viruses were administered to volunteers to determine the level of virulence of wild-type virus for comparison with that of attenuated experimental vaccines and to use as a challenge inoculum to assess the level of protection afforded by these vaccines. Daily nasopharyngeal washes were obtained on each volunteer with 16 ml of veal infusion broth.

Infectivity and interferon determinations. Influenza virus and Sindbis virus infectivity titers were determined as mean tissue culture infectious dose by assaying for hemadsorption or cytopathic effect in primary rhesus monkey kidney and primary chicken embryo fibroblast cultures, respectively. Interferon sensitivities of the influenza and Sindbis viruses were determined as the minimum number of reference units per milliliter which significantly ($0.5 \log_{10}$) inhibited infectivity yields. Initially the titer of a human leukocyte interferon in reference units was determined by comparing its titer with that of human reference interferon NIH GO23-901-527. HEK roller tube cultures were exposed to 0.5

\log_{10} dilutions of human leukocyte interferon for 24 h at 37 C. The culture tubes were then washed and inoculated with the indicated viruses at a multiplicity of infection of 0.01. Two hours postinoculation each tube culture was washed four times to remove residual unadsorbed virus and then was incubated with serum-free Eagle no. 2 medium at 37 C. The influenza-inoculated cultures were harvested for infectivity yield assay at 24 h postinfection; the Sindbis cultures were harvested at 48 h.

The quantity of interferon (reference units per milliliter) in the NP wash specimens was determined either by a yield reduction of Sindbis virus in human foreskin fibroblasts or by the method of inhibition of vesicular stomatitis virus-induced cytopathic effect as previously described (1, 10). The virus present in the NP wash specimens was either inactivated by addition of specific antisera (7) or by treatment of samples at pH 2.0 for 24 h (for the Udorn/72 and Georgia/74 viruses) (9).

RESULTS

Sensitivity of influenza strains to interferon. Influenza A/Bethesda/1968 (H3N2), a representative strain of the early "Hong Kong" subtype, previously was shown to be relatively sensitive to interferon (7). The interferon sensitivity of this strain in comparison with two more recently appearing antigenic variants of the H3N2 subtype was examined to determine if the high level of interferon sensitivity of the 1968 virus was a general property shared by subsequent H3N2 viruses. The interferon sensitivities of the 1968, 1972, and 1974 strains were comparable, if not somewhat greater than that of the known sensitive Sindbis virus in several experiments. Table 1 shows the results of a representative experiment. Other experiments using different multiplicities of infection (0.01 to 1.0) demonstrated similar levels of interferon sensitivity.

Production of interferon during infection of volunteers. Adult seronegative volunteers were administered influenza A virus intranasally and the daily NP washes of these volunteers were examined quantitatively for the presence of virus and interferon (Fig. 1). Those volunteers who exhibited evidence of infection as

TABLE 1. Interferon sensitivity of three antigenic variants of influenza A (H3N2) virus and of Sindbis virus

Virus	No. of reference interferon units required for 0.5 \log_{10} reduction of virus yield
A/Bethesda/1015/1968	0.1
A/Udorn/307/1972	1.0
A/Georgia/101/1974	0.2
Sindbis	1.0

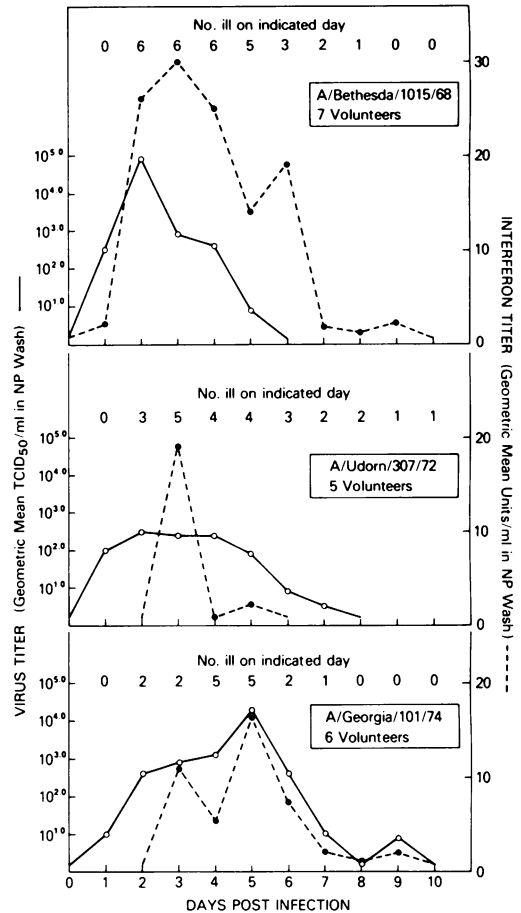


FIG. 1. Response of volunteers to the intranasal administration of three strains of influenza A (H3N2) virus.

defined by either viral shedding or a fourfold or greater rise in serum hemagglutination inhibition antibody were included in this study. Slight variations among the three viruses were observed. The 1968 virus appeared to be slightly more virulent with higher peak titers of interferon and virus. For three of the six volunteers receiving the 1974 virus the onset of illness, virus shedding, and interferon response appeared to commence on day 3, rather than on day 1 or 2. Nevertheless, a general pattern emerges for the three antigenic stains. Virus shedding was usually observed on day 1. Illness and interferon appear on day 2, 3, or 4 as the peak virus titer was attained. Because the method of obtaining nasopharyngeal wash specimens results in an approximate 20-fold dilution of the secretions, the mean peak interferon titers were actually 300 to 600 units/ml in the secretions, and individuals' peak titers

(i.e., corrected for the 20-fold dilution) exceeded 10,000 units/ml in several instances. Figure 1 represents a composite of the kinetics of the development of illness, interferon production, and virus shedding in each study. In addition, each individual's response was analyzed for the relative times of appearance of illness, initial and peak virus shedding, and initial and peak interferon shedding. A summary of the observations made on the 14 volunteers who became ill is presented in Table 2. Virus initially appeared on the average 1.3 days postinoculation. Next to appear were illness and initial interferon responses at 2.4 days. Peak virus titers were measured one-half day later at 2.9 days. Finally, the peak interferon response was attained at 3.4 days. The excellent correlation between the amplitudes of the peak virus and interferon titers for each volunteer, which was previously demonstrated for the 1968 strain, also was apparent for the 1972 and 1974 strains (data not shown) (7).

DISCUSSION

The data presented indicate that three antigenic variants of the influenza A H3N2 subtype isolated in different years and in different geographic locations exhibited a similar degree of high sensitivity to human interferon and a similar ability to induce nasopharyngeal interferon. These data suggest that these properties might be generally applicable to the viruses within the H3N2 subtype and that future strains of human influenza A viruses within this subtype will also exhibit similar interferon sensitivity and ability to induce interferon. It is of interest that those ribonucleic acid respiratory viruses which have been studied for interferon sensitivity were found to be quite sensitive (4, 7, 9), whereas deoxyribonucleic acid respiratory viruses were relatively insensitive (3, 11).

TABLE 2. Average time of appearance of parameters of infection after the administration of a wild-type influenza A virus (14 volunteers)^a

Parameter	Avg days of appearance postinfection ± SE
Initial virus shedding	1.3 ± 0.21
Initial interferon shedding	2.4 ± 0.25
Initial symptoms of illness	2.4 ± 0.23
Peak NP virus titer	2.9 ± 0.17
Peak NP interferon titer	3.5 ± 0.27

^a All values differ significantly from each other except initial interferon shedding and initial symptoms of illness ($P < 0.05$, Student's *t*-test). SE, Standard error of the mean.

Since interferon or interferon inducers have been developed as a possible approach to the prevention and therapy of influenza viral illness, it is important to know that one can reasonably expect subsequent H3N2 strains to exhibit a high level of sensitivity to interferon. For those who would use interferon prophylactically, these results are encouraging. However, they are less encouraging for therapeutic use. The appearance of the first indications of illness and of a measurable interferon response were coincident, suggesting that once symptoms have appeared the opportunity for therapeutic intervention is quite limited. The peak virus shedding, the development of symptoms, and the peak interferon response all occur within a 1.1-day period. Since the endogenous interferon in respiratory secretions is substantial at an early time, it is unlikely that induction or administration of additional interferon after illness has begun will alter the course of disease.

Only 0.5 days after symptoms and interferon appeared virus shedding had begun to decrease by undefined mechanisms. A chemotherapeutic agent, therefore, would have to be effective against high, peak concentrations of virus and act in addition to the host's interferon and other recovery mechanisms already in operation. Perhaps this partially explains the limited therapeutic effectiveness of amantadine in experimental human influenza A virus infection (12).

The quantitative relationships between illness, virus shedding, and interferon response have not been evaluated during the more severe forms of influenzal illness in adults, such as pneumonitis, and also have not been evaluated in children undergoing influenzal illness. It is possible that a prolonged period of high level virus shedding or a delay in the interferon response (as was observed with parainfluenza type 1 virus infection in adult volunteers [9]) may occur. Therefore, the limitations of antiviral intervention that might exist for adults undergoing mild experimental influenza might not apply to other forms of influenza and to other respiratory virus infections.

LITERATURE CITED

1. Armstrong, J. A. 1971. Semi-micro, dye-binding assay for rabbit interferon. *Appl. Microbiol.* 21:723-725.
2. Baron, S. 1963. Mechanism of recovery from viral infection. *Adv. Virus Res.* 10:39-64.
3. Gallagher, J. G., and N. Khoobyarian. 1972. Adenovirus susceptibility to human interferon during one-step replication. *Infect. Immun.* 5:905-908.
4. Hill, D. W., S. Baron, and R. M. Chanock. 1969. Sensitivity of common respiratory viruses to an interferon inducer in human cells. *Lancet* 2:187-188.

5. Isaacs, A. 1963. Interferon. *Adv. Virus Res.* 10:1-38.
6. Knight, V. 1964. The use of volunteers in medical virology. *Prog. Med. Virol.* 6:1-26.
7. Murphy, B. R., S. Baron, E. G. Chalhub, C. P. Uhlen-
dorf, and R. M. Chanock. 1973. Temperature-sensi-
tive mutants of influenza virus. IV. Induction of
interferon in the nasopharynx by wild-type and a
temperature-sensitive recombinant virus. *J. Infect.*
Dis. 128:488-493.
8. Murphy, B. R., E. G. Chalhub, S. R. Nusinoff, and
R. M. Chanock. 1972. Temperature-sensitive mutants
of influenza virus. II. Attenuation of ts recom-
binants for man. *J. Infect. Dis.* 126:170-178.
9. Murphy, B. R., D. D. Richman, E. G. Chalhub, C. P.
Uhlen-
dorf, S. Baron, and R. M. Chanock. 1975.
Failure of attenuated temperature-sensitive influ-
enza A (H3N2) virus to induce heterologous inter-
ference in humans to parainfluenza type 1 virus.
Infect. Immun. 12:62-68.
10. Oie, H. K., C. E. Buckler, C. P. Uhlen-
dorf, D. A. Hill,
and S. Baron. 1972. Improved assays for a variety of
interferons. *Proc. Soc. Exp. Biol. Med.* 140:1178-1181.
11. Schacter, N., M. A. Gallin, M. Weissenbacher, S.
Baron, and A. Billiau. 1970. Comparison of anti-
viral action of interferon, interferon inducers, and
IDU against herpes simplex and other viruses. *Ann.*
Ophthalmol. 2:795-798.
12. Togo, Y., R. B. Hornick, and A. T. Dawkins, Jr. 1968.
Studies on induced influenza in man. I. Double-
blind studies designed to assess prophylactic efficacy
of amantadine hydrochloride against a 2/Rockville/1/
65 strain. *J. Am. Med. Assoc.* 203:1089-1094.