THE ROLE OF INTERFERON IN VACCINIA VIRUS INFECTION OF MOUSE EMBRYO TISSUE CULTURE

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Interest in the interference phenomena is currently focused on interferon, a soluble product of influenza virus-infected cells, capable of interfering with replication of a wide range of viruses (1, 2). Utilizing a number of different cell-virus systems other investigators have reported the production of related interferon-like substances with the viruses of polio (3, 4) and measles (5) in human amnion cells, myxoviruses in MCN cells (6), para-influenza 3 virus in KB cells (7), and Sindbis virus in chick embryo cells (8). The possible role of interferon in a number of biological phenomena has been discussed. Ho and Enders (4) demonstrated that the initiation and maintenance of a chronic infection with poliovirus in human amnion cell cultures was partially dependent upon production of interferon. Henle (6) and coworkers have reported the production of an interferon in a myxovirus-MCN carrier culture. In a para-influenza carrier culture Chany (7) postulated that the interferon contributed to the establishment of the carrier state, but was less important in maintaining that cell-virus relationship. Baron and Isaacs (9) have recently reviewed the evidence which suggests that interferon may play a role in the natural recovery process from viral infections. *In vivo* it has been demonstrated that in two types of viral infections—influenza in mouse lung (10) and O'ynong-ynong in mouse brain (11), the recovery process begins before the appearance of antibodies. In both instances, demonstrable levels of interferon are present early and may be correlated with the initiation of the recovery process.

The present study reports an interferon-like substance produced by vaccinia virus in mouse embryo cells in tissue culture. The physical properties of this substance are defined and its role in the initiation, maintenance, and recovery of a chronic vaccinia infection *in vitro* is described.

Materials and Methods

Virus.—A calf lymph strain of vaccinia virus maintained by passage in monkey kidney cultures was obtained from Dr. Joel Warren. The virus pool was prepared in HeLa cells and titered 2×10^6 plaque-forming units (pfu) per ml. Sindbis virus was obtained from Dr. Phillip Russell, Walter Reed Army Medical Center, Arbor Virus Unit, as second mouse brain passage of an isolate from a Malayan mosquito pool. The Sindbis virus pool was grown in an established mouse embryo cell line and titered approximately 10^7 pfu per ml.

Cells.—ME-29 is an established line of mouse embryo cells derived from a C57Bl mouse, and is now in the 75th subculture. Cultures were grown and maintained in Eagle's medium with 5–10 per cent calf serum.

3-B is an established line of mouse embryo cells cloned from a polyoma virus carrier culture. The cell line originated from an NIH random-bred Swiss mouse and has been grown and maintained in Eagle's medium with 5–10 per cent horse serum. These cells have remained polyoma-free for 68 subculture passages since cloning.

Virus Titration.—Sindbis virus was titered by plaque method in ME-29 cells. Virus was adsorbed for 1½ hours and overlaid with agar medium. A second agar medium overlay containing 1:30,000 neutral red was made at 24 hours and plaque counts made at 48 and 72 hours.

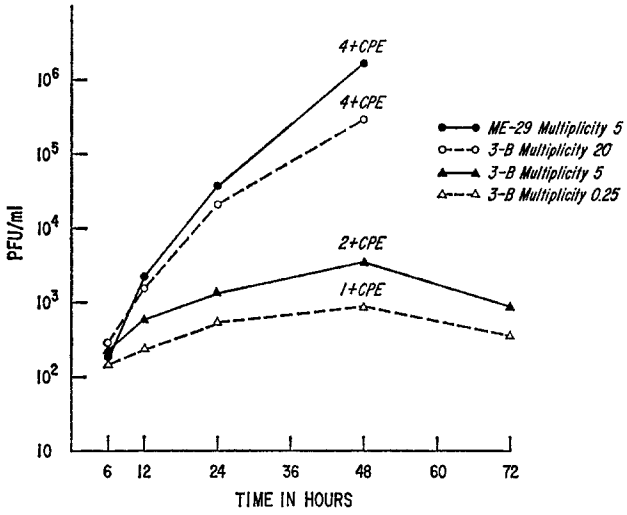

FIG. 1. Multiple cycle growth curves of vaccinia virus in resistant 3-B cells challenged with varying input multiplicity compared to a susceptible mouse embryo line (ME-29).

Vaccinia virus was also quantitated by plaque method in HeLa cells with a 3 hour adsorption followed by agar medium overlay. A second overlay with 1:30,000 neutral red was made on the 3rd day and counts from the 4th to 6th days.

Agar medium used for plaque assay contained 0.95 per cent agar in Earle's BSS with 0.1 per cent bovine albumin and 0.1 per cent yeast extract.

RESULTS

During the course of studies of the interference phenomenon a cloned line of mouse embryo cells, 3-B, was observed to be moderately resistant to vaccinia virus. The nature of this resistance was investigated. It was noted that the resistance was partially dependent upon the input multiplicity of the challenge virus. Cultures challenged with a multiplicity of 10 to 20 pfu per cell developed typical vaccinia cytopathic effect (CPE) within 18 to 24 hours, and by 48 hours the culture was destroyed. When the multiplicity was reduced to 0.25 to 5,

pock-like areas of CPE appeared after the same interval, but remained circumscribed and did not progress beyond 2+, (approximately 25–50 per cent of the cell sheet). This resistance could also be demonstrated by the reduced efficiency of virus production in 3-B cells challenged with a low multiplicity (Fig. 1). These data, along with other evidence to be presented, suggested the possibility that production of an interfering substance by infected cells during the first 1 to 2 cycles of virus replication reduced the susceptibility of the remaining uninfected cells in the culture. When fluids were harvested from the vaccinia-infected 3-B cultures and were centrifuged to remove the infectious virus particles, the supernatant did in fact contain an inhibitory substance which shares many physical characteristics with previously reported interferons. Results of

TABLE I

Interferon Activity in Fluids of Resistant 3-B Cells Infected with Vaccinia Virus

Exp. No.	CPE*	Challenge virus	Percentage plaque reduction‡
			per cent
1	1 to 2+	Vaccinia	94
2	2+	Vaccinia	88
3	2+	Sindbis	75
4	2+	Sindbis	68

* At 72 hours in original vaccinia-infected 3-B culture supplying the interferon.

‡ Plates were pretreated with 1 cc interferon diluted 1:3 in Eagle's medium for 18 hours. Figure represents per cent of the number of plaques in control cultures.

several experiments in which the interferon activity of the harvests was assayed are presented in Table I.

Production of Interferon.—

In the initial experiments interferon preparations were obtained by inoculation of the 3-B cell line with infective vaccinia virus. 1 liter Blake bottles containing about 5×10^6 cells were infected with approximately 10^6 pfu of vaccinia virus. CPE appeared at 24 hours as circumscribed pocks which gradually enlarged and increased in number over the next 24 to 36 hours. By 72 hours further progress was arrested and some regrowth of cells into the areas of CPE was observed. At this time fluids were harvested, centrifuged at 2000 RPM for 10 minutes to remove cell debris, and then at approximately 50,000 *g* for 45 minutes. Supernates were stored at 4°C and were the source of the interferon.

At a later stage of these investigations it was observed that supernates of cultures inoculated with ultraviolet-inactivated vaccinia demonstrated greater interferon activity than did those from live virus–infected cultures. Vaccinia virus was inactivated in a Habel-Sockrider ultraviolet apparatus (12). 5 ml of a completely inactivated virus preparation which had originally contained

2.5×10^6 pfu/ml was inoculated into a 1 liter Blake bottle of 3-B cells. Fluids were harvested at 40 to 48 hours and interferon preparations made as described above.

Assay of Interferon.—

Interferon was assayed by a plaque reduction method. Monolayers of ME-29 cells in plastic plates were pretreated for 16 to 20 hours with 3 ml of the dilutions of the interferon preparation being tested. This was removed and a 0.5 ml challenge inoculum of Sindbis virus containing approximately 150 pfu was allowed to adsorb for 1½ hours. The monolayer was then overlaid with agar medium. A second overlay containing neutral red was added at 24

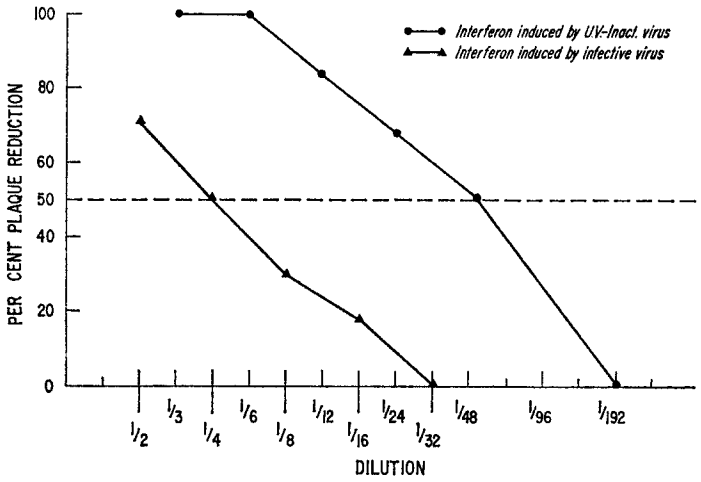

FIG. 2. Titer of interferon activity in harvests of 3-B cultures inoculated with infective or ultraviolet-inactivated vaccinia virus. Interferon activity is tested against Sindbis virus by plaque reduction method.

hours and plaque counts were made at 48 and 72 hours. In a number of experiments vaccinia was used as the challenge virus. ME-29 cells were pretreated with interferon as described above. The inoculum of vaccinia virus containing approximately 150 pfu was removed after a 3 hour adsorption period and cultures were incubated in Eagle's medium with 10 per cent calf serum. When plaques became visible, usually between 24 and 30 hours after inoculation, the fluid medium was removed and the cell monolayers were overlaid with agar medium containing 1:50,000 neutral red. Interferon activity was measured as the percentage reduction in plaque count in the interferon-treated plates as compared with control plates treated with a similar dilution of spent media harvested from uninfected 3-B cultures.

Fig. 2 shows the results of a quantitative assay of the interferon activity in harvests from cultures inoculated with infective and ultraviolet-inactivated virus.

Properties of Vaccinia Interferon.—The properties of the interfering substance were shown to be similar to that reported for other interferon-like substances.

Sedimentability: The interferon remained in the supernatant following ultracentrifugation at 90,000 *g* for 1 hour.

Heat Stability: Interferon activity remained after heating for 60 minutes at 60°C. Most of the activity was destroyed, however, when preparations were heated at 80°C for 60 minutes.

pH Stability: The activity of the preparation was not altered by treatment for 18 hours at pH 3.

Dialysis: Vaccinia interferon did not diffuse through the membrane when dialyzed for 24 hours against a balanced salt solution at 4°C.

Trypsin: Treatment with 0.1 per cent trypsin at 37°C for 1 hour destroyed 90 per cent of the inhibitory activity.

Ether: Mixture with ether at 4°C for 2 hours destroyed approximately 90 per cent of the activity.

TABLE II

Sensitivity of Other Viruses to Vaccinia Interferon As Determined by Plaque Reduction Method in ME-29 Cells

Virus	Interferon dilution	Percentage plaque reduction
		per cent
Vaccinia	1:6	100
	1:12	95
Sindbis	1:6	100
	1:12	80
Encephalomyocarditis	1:3	98
Herpes simplex	1:3	94
Eastern equine encephalitis	1:3	90
Vesicular stomatitis	1:3	53

Lack of Association with Hemagglutinin: The interferon preparation demonstrated no hemagglutinating properties. The soluble hemagglutinin of a normal vaccinia virus pool was sedimented by ultracentrifugation at 50,000 *g* for 2 hours, whereas a similar sediment of the interferon preparation demonstrated no hemagglutinin.

Antigenicity: Incubation of the interferon for 2 hours with vaccinia hyperimmune rabbit serum caused no loss of activity.

Viral Specificity.—The data presented in Table II demonstrate the lack of viral specificity of vaccinia interferon. Although all of the viruses tested were inhibited, definite differences in sensitivity were noted. It is interesting that herpes simplex virus which is only moderately sensitive to influenza interferon (13) is readily inhibited by vaccinia interferon. In contrast, vesicular stomatitis virus which would appear to be relatively sensitive to other interferons (6, 8) was the least sensitive of the viruses tested. Further studies are indicated to determine whether such differences: (*a*) are a function of the virus-cell system used for the assay, (*b*) depend on a variation in sensitivity among strains of the same virus, or (*c*) reflect actual differences in the activity of viral interferons.

Cell Specificity.—The vaccinia interferon prepared in mouse embryo cells was tested for its ability to inhibit the multiplication of vaccinia virus in heterologous cell lines. Activity was assayed by plaque reduction method with vaccinia as the challenge virus. Interferon produced in 3-B mouse embryo cells was effective in all mouse cell lines studied, but not in primary monkey kidney, chick embryo, or rabbit kidney cultures. The exception to this species specificity was in the HeLa cell line carried in our laboratory. Our data suggest that the interferon is partially effective in HeLa cells. The differences between interferon-treated as compared to control HeLa plates were of borderline significance, but were consistently present. (Table III). This effect of mouse interferon in a cell line of human origin confirms previous evidence (14, 15) that species specificity is not absolute. Furthermore, it provides additional evidence (16) of the activity of interferon in some strains of malignant cells.

TABLE III

Effect of Interferon in HeLa Cells

Exp. No.	Control*	Interferon*	Reduction	p value‡
			per cent	
1	54.7	31.7	42.5	<0.01
2	238	171	28	<0.01
3	62.2	52	16	=0.12

* Average number of vaccinia plaques per plate in control and interferon-treated groups.
‡One-tailed t test.

Interferon Production and Cell Resistance in Different Cell Lines.—The frequency with which interferon was a product of vaccinia virus infection in other cell lines was investigated. Interferon was assayed in the fluids of replicate cultures of three different mouse cell lines challenged with vaccinia virus. Production of interferon was demonstrated in each of the vaccinia virus–mouse cell systems studied; however, quantitative differences were apparent. The results given in Table IV suggest an inverse relationship between the susceptibility to CPE of mouse cell lines and their capability of producing interferon.

Vaccinia Carrier Culture.—As noted before, when the resistant 3-B culture was inoculated with a multiplicity of vaccinia virus varying from 0.25 to 5, CPE was incomplete. If the culture was maintained, virus replication continued, spread of CPE was controlled, and new cell growth could be observed in the pock-like areas of cell destruction—a carrier culture. It became apparent, however, that the balance of factors necessary to maintain the carrier culture was extremely delicate. During the course of our observations all of the theoretically possible outcomes of such an equilibrium have been demonstrated: (*a*) imbalance in favor of the infection with rising virus titer and destruction of

the culture, (*b*) maintenance of the equilibrium with persistence of the carrier state, and (*c*) imbalance in favor of the factors inhibiting the spread of the virus with the culture eventually being cured of its vaccinia infection.

TABLE IV

Relationship Between Ability to Produce Interferon and Susceptibility to CPE by Vaccinia Virus in Three Different Mouse Cell Cultures

Cell line	Interferon production*	Susceptibilty‡
	per cent	
Primary mouse embryo	50	2×10^2 pfu
ME-29	50	2×10^2 pfu
3-B	95–100	2×10^4 pfu

* Per cent plaque reduction of a 150 pfu challenge of vaccinia virus by the interferon preparation produced in the indicated cell line.

‡ No. of pfu of vaccinia virus pool required to produce CPE in the indicated cell line.

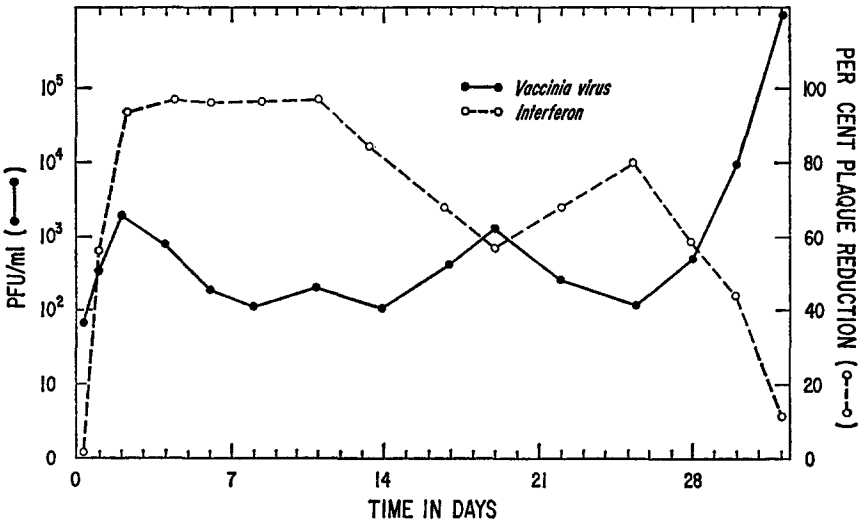

FIG. 3. Balance between interferon and virus production in a vaccinia carrier culture in 3-B cells. Virus titers are pfu/ml of supernatant and interferon quantitation is the per cent reduction of 150 pfu of vaccinia by 1 cc of the same samples.

Studies were initiated to quantitate the role of interferon in this virus-cell equilibrium. Fig. 3 illustrates an experiment in which virus and interferon titers were determined over a 5 week period following initiation of vaccinia infection in the resistant cell culture. This experiment is an example of an outcome in favor of the virus with eventual destruction of the culture and fall of the interferon titer.

Effect of Manipulation on Virus-Interferon Equilibrium.—Attempts were made to alter the equilibrium in the carrier culture.

Three replicate cultures of 3-B cells were challenged with approximately 0.5 pfu of vaccinia virus per cell. Following the initial growth cycles with establishment of the interferon-virus balance, one culture was trypsinized every 2nd day, one was subjected to media changes every 2nd day, while the third was changed at 4 day intervals. Approximately 10–20 per cent of the spent medium was allowed to remain in the third culture at the time of the medium change. Frequent trypsinization rapidly shifted the balance in favor of the virus with destruction of the culture and rising virus titers. Frequent changes of the medium had the same effect. In

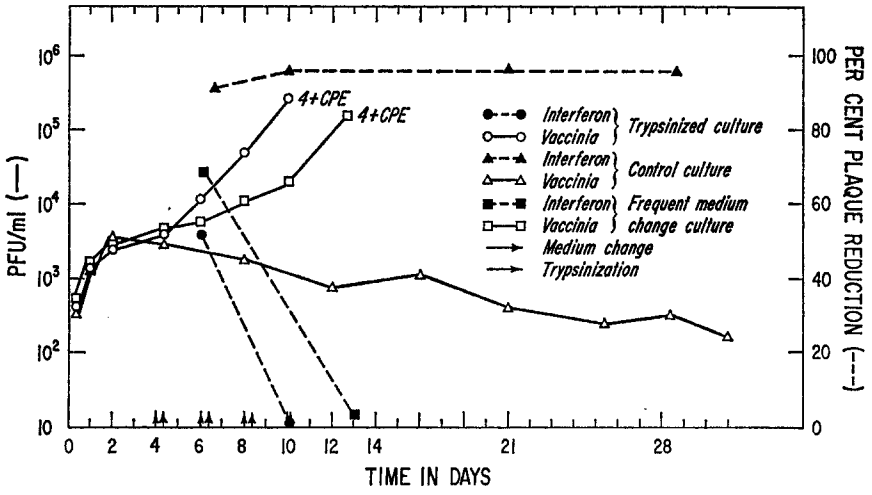

FIG. 4. Enhancement of virus production and CPE by procedures detrimental to interferon. Vaccinia virus titers are pfu/ml and interferon quantitation is the per cent reduction of 150 pfu of vaccinia virus by 1 cc of the sample.

contrast, the culture that was handled in such a way as to minimize the dilution factor and to preserve the interferon was maintained as a carrier culture.

Fig. 4 confirms the inverse relationship between virus production with CPE and interferon production with cell protection.

Maintenance of the Carrier Culture.—The carrier state which developed following vaccinia infection of a 3-B culture was moderately unstable. The equilibrium between virus and interferon could be maintained by cognizance of the effect of handling and media changes. If CPE appeared to be progressing and the virus titer rose, the culture was changed less frequently and 10–20 per cent of the interferon containing spent medium was left in the bottle at the time of the change. On the other hand, if CPE seemed to be clearing and the virus titer fell, complete media changes were made more frequently. Under such conditions vaccinia carrier cultures could be maintained for 3 to 5 month periods. The cultures which were maintained for this length of time were lost as a result of technical failure and not from imbalance of the virus-interferon equilibrium.

Recovery from Vaccinia Infection.—In several experiments when the carrier culture was maintained, under conditions which favored the preservation of the interferon, CPE gradually cleared and the virus was eventually eliminated from the culture. This spontaneous recovery was observed to occur from 4 weeks to 3 months after the initiation of infection.

DISCUSSION

The studies presented demonstrate the production of an interferon-like substance in several lines of mouse embryo cells by a DNA virus, vaccinia. This substance is shown to have physical properties similar to those of influenza interferon and other reported interferon-like substances.

Vaccinia interferon appears to be primarily responsible for the partial resistance of the cloned line of mouse cells to vaccinia CPE and for the establishment and maintenance of a vaccinia carrier culture.

Considerable evidence has accumulated which suggests that interferon may play an important role in the natural recovery from *in vivo* viral infections. The data presented demonstrate the spontaneous recovery of a cell culture from vaccinia virus infection. Evidence suggests that this recovery was mediated by interferon. Thus a model for recovery from a virus infection is provided in an *in vitro* system where immunologic mechanisms were not operative.

Mechanical manipulation which diluted, or enzyme treatment which destroyed interferon led to the disruption of the equilibrium between virus and interferon in the carrier culture, resulting in a rising virus titer and eventual complete CPE. Such manipulation might prove useful in virus isolation or adaptation procedures where production of interferon could be the factor responsible for minimal CPE and low virus titers.

The data presented add to the evidence suggesting that the production of interferon may be a general property of many virus-cell systems. The effect of interferon production may be minimal as in the primary mouse and ME-29 lines, or it may be an important factor in the resistance of a culture as in the 3-B line. In the susceptible cell lines interferon was produced in low titers, and apparently was able to inhibit virus replication only when the inoculum contained a low multiplicity of vaccinia virus, less than 0.001 pfu/cell. In contrast, the resistant 3-B line produced higher titers of interferon when infected with vaccinia virus and was resistant to a 100-fold larger virus challenge. Studies in progress suggest that this cell line also produces interferon when infected with other viruses. The question arises as to why this cell line has an enhanced ability to respond to virus infection with the production of interferon. It is interesting to speculate: (*a*) that in the development of the original polyoma carrier culture, from which the 3-B line was derived, selection of a cell more efficient in interferon production occurred, or (*b*) that a step in the process of malignant transformation induced by the polyoma virus might involve the greater capability for interferon production.

SUMMARY

The production of an interferon-like substance by vaccinia virus is described.

The physical properties of vaccinia interferon are shown to be similar to those of previously reported interferons.

Data defining the role of vaccinia interferon in cell resistance and in establishment and maintenance of a carrier culture are presented.

Elimination of virus from an infected culture is demonstrated and the role of interferon in the recovery process is considered.

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