*A HUMAN INTERFERON THAT CROSSES THE SPECIES LINE**

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Interferons prepared in cells of one animal species have been characterized by activity specific for the species in which the interferon was prepared. They may exert a considerable effect in cells of closely related species. Crude interferons also show minor degrees of cross-reactivity with cells of "unrelated" species, but this has been ascribed to inhibitors other than interferon and high heterospecific activity has not been reported. The aim of the present report is to describe the preparation and characterization of a human interferon that is more active in rabbit cells than in human fibroblasts.

Materials and Methods.—Tissue cultures: The method of preparation of primary cultures has been described.[1] Human foreskin and fetal fibroblasts were passaged by splitting monolayers at a 1:3 ratio and used between passages 10 and 20. The growth medium was Eagle's medium supplemented with sodium bicarbonate, 0.75 gm/liter; fetal bovine serum, 10%; penicillin, 100 U/ml; kanamycin, 100 μg/ml; and mycostatin, 50 U/ml. In maintenance medium, 1.5 gm/liter of bicarbonate and fetal bovine serum, 2%, were substituted. Agar overlays, 4 ml per 6-cm plastic Petri dish, contained the same constituents as the growth medium plus Bacto-agar, 1.2%, and neutral red, 25 mg/liter. The bicarbonate content in the medium of all cultures incubated in a 5% CO_2 atmosphere was 2.25 gm/liter. The incubation temperature was 37°.

Viruses: Newcastle disease virus (NDV) (California strain, American Type Culture Collection) was obtained from the allantoic fluid harvested 2 days after infection of 11-day-old chick embryos, and assayed by plaque formation on chick embyro cells. Vesicular stomatitis virus (VSV), Indiana strain, was propagated in green monkey kidney cells and assayed by plaque formation in the different cell types used for interferon assay. Vaccinia virus, WR strain, was propagated in rabbit kidney cells and assayed by plaque formation in the same cells. Rubella virus from the tissues of an infected fetus was passed once in BHK-21 cells and assayed by a modified hemadsorption-negative plaque method.[2] Mengovirus (S variant, courtesy of Dr. J. S. Colter) was propagated in rabbit kidney cells and assayed by plaque formation in the same cells; the agar overlay contained no serum, but included skim milk, 0.5%, and protamine sulfate, 300 mg/liter.

Production of interferon: Interferon was prepared by inoculating foreskin fibroblast monolayers in 16-oz (80 cm^2) bottles with 1–3 plaque-forming units (PFU) of NDV per cell contained in 30 ml of maintenance medium without serum. The fluids were harvested after 48 hr of incubation in a 5% CO_2 atmosphere. This procedure was similar to that described by Merigan *et al.*[3] Pooled fluids were acidified at pH 2.0 with HCl, stored 5 days at 4°, brought to pH 7 with NaOH, subjected to low-speed centrifugation, and then centrifuged twice for 2 hr at 100,000 *g*. These preparations were stored frozen at −20°. The same method was used to produce rabbit interferon in rabbit kidney cells. Rabbit serum interferon was produced as described.[4]

Assay of interferon: Interferon assays were made on monolayers of a strain of foreskin fibroblasts in Petri dishes. Four ml of interferon diluted in maintenance medium were left in contact with the cells for 18 hr in a 5% CO_2 atmosphere. The cells were then washed once with medium without serum, and 50–80 PFU of VSV in 0.2 ml were allowed to adsorb for 1 hr, after which agar overlay was applied. This procedure was similar to that used by Merigan *et al.*[3] Plaques were counted after 2 or 3 days, i.e., 1 day after the

first macroscopic evidence of plaque formation. Interferon titers are expressed as the highest twofold dilution giving at least 50% plaque reduction. This dilution is said to contain one 50% plaque-reducing dose, or PR_{50}-unit, of interferon. Two or three replicate plates were used for each interferon dilution tested. Assays for interferon-like activity on other cells were conducted in the same fashion. Interferon activity was considered absent when, at the lowest dilution tested, usually 1/8, less than 50% reduction of the number of VSV plaques occurred. For pretreatment of rabbit kidney cells with dactinomycin, the antibiotic was diluted in medium 199, incubated on the cells for 1 hr at pH 7.2 and 37°,[5] in vol of 1 ml for tube cultures and 2 ml for Petri dishes. Cells were washed 4 times, and further treated with interferon and challenge virus as described.

Results.—Production and activity of interspecific factor (ISF): The titer of three interferon lots prepared in human foreskin cells was 1/128–1/512 on cells from the same strain (human interferon). The corresponding interspecific activity on rabbit kidney cells was 1/4096–1/8192 (interspecific factor). The differences observed between the three preparations were not significant (Table 1). The use of different lots of NDV and of rabbit kidney cells, and different passage numbers of foreskin cells for production or assay resulted in essentially the same titers of human interferon and ISF activity. The preparations, clarified by high-speed centrifugation, provided stock interferon for further study. Control fluids were prepared in the same manner, omitting virus or using uninfected allantoic fluid. They failed to inhibit VSV plaque formation either on human foreskin or on rabbit kidney cells. Fluids containing NDV, when processed without incubation on foreskin cells, were equally inactive.

A second strain of foreskin cells and a strain of human fibroblasts derived from skin and muscle of a three-month fetus also produced ISF. The human interferon activity and the ISF activity were of the same order as those shown in Table 1. A human interferon produced in human lung fibroblasts with ultraviolet-inactivated NDV[1] and an interferon produced in human leukocytes with live NDV[6] having titers of 1/80 and 1/57, respectively, failed to show ISF activity on rabbit kidney cells. Also, a human serum with an interferon titer of 1/16 as a result of live measles virus vaccination, and a human urine with a titer of interferon-like activity of 1/16, failed to reduce VSV plaque counts on rabbit kidney cells. Rabbit interferon was not reciprocally active in human cells. Interferon prepared *in vitro*, with a titer of 1/512, and serum interferon, titer 1/256 in rabbit kidney cells, failed to reduce VSV plaque formation in human foreskin cells.

Stock interferon reduced VSV plaque formation in primary rabbit embryo fibroblasts by 50 per cent at a 1/1024 dilution. However, no reduction was ob-

TABLE 1. *Activity of interferon prepared in human foreskin cells and assayed in human foreskin and rabbit kidney cells.*

Interferon lot	Titer before Centrifugation*		Titer after Centrifugation*	
	Human cells	Rabbit cells	Human cells	Rabbit cells
1	512†	8192	256	8192
			256	4096
2	256	Not done	128	4096
			512	4096
3	Not done	4096	256	>8192
		8192	128	4096

* Two times, 2 hr at 100,000 *g*.
† PR_{50}-units, see text.

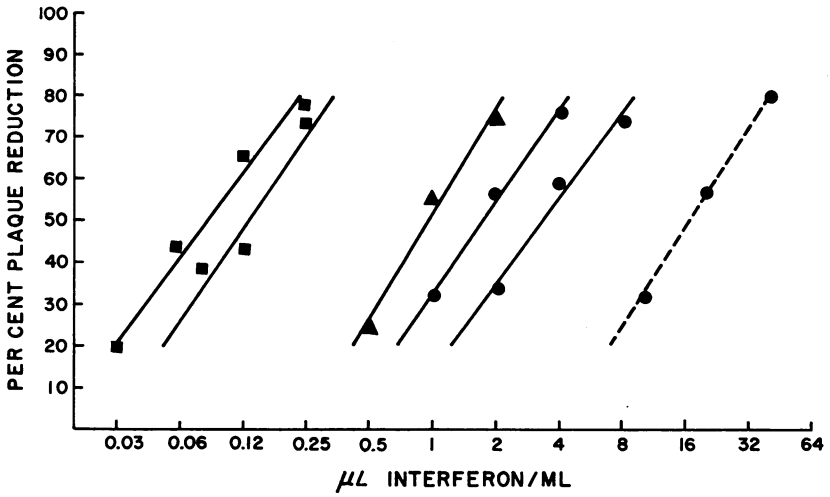

FIG. 1.—Dose response of interferon obtained by NDV infection of: (*a*) human foreskin fibroblasts (*continuous line*), and assayed by VSV plaque reduction in human foreskin fibroblasts (●), rabbit embryo fibroblasts (▲), and rabbit kidney cells (■); (*b*) human leukocytes (*dotted line*), assayed similarly on foreskin cells (●); no activity on rabbit cells was detected.

served after treatment of 3-day-old primary fibroblast monolayers of 11-day chick embryos or in hamster embryo fibroblasts. The interferon titer on human embryonic kidney cells was 1/128.

Interferon properties of interspecific factor: The factor was resistant to acid treatment, and was not sedimentable by high-speed centrifugation (Table 1). Stock interferon did not inactivate VSV. The same number of PFU were recovered after incubation of the virus for one hour at 37° with stock interferon and with maintenance medium. Dialysis against 100 volumes of saline for 18 hours at 4° failed to reduce both human interferon titer and the ISF activity of the standard interferon. Titers of 1/256 and 1/4096, respectively, were recovered. Treatment with 0.1 per cent trypsin (Difco 1:250) at pH 7.4 for one hour at 37° resulted in a complete loss of both human interferon and ISF activity. Resistance to thermal inactivation was shown by recovery of ISF activity after 30 minutes at 56°, with the titer remaining constant at 1/4096; the human interferon activity was 1/128 after heating. After six days at 37°, the ISF titer was still 1/1024. The interferon activity could not be removed by washing pretreated cells. Cells were treated overnight with interferon at different dilutions. Then the monolayers were washed four times and VSV challenge added. The ISF and human interferon titers were the same in washed and unwashed cultures.

The dose response of ISF and human interferon is analyzed in Figure 1. When plaque reduction is plotted against interferon concentration on a logarithmic scale, an S-shaped curve is obtained and the intermediate portion of the curve, which is plotted, is linear.[7] The slopes of the activity curves of the stock interferon on human and rabbit cells are identical. The slopes are also identical to that of the activity of a human leukocyte interferon which was devoid of activity on rabbit cells. Since the slopes were identical, there was no indication that the

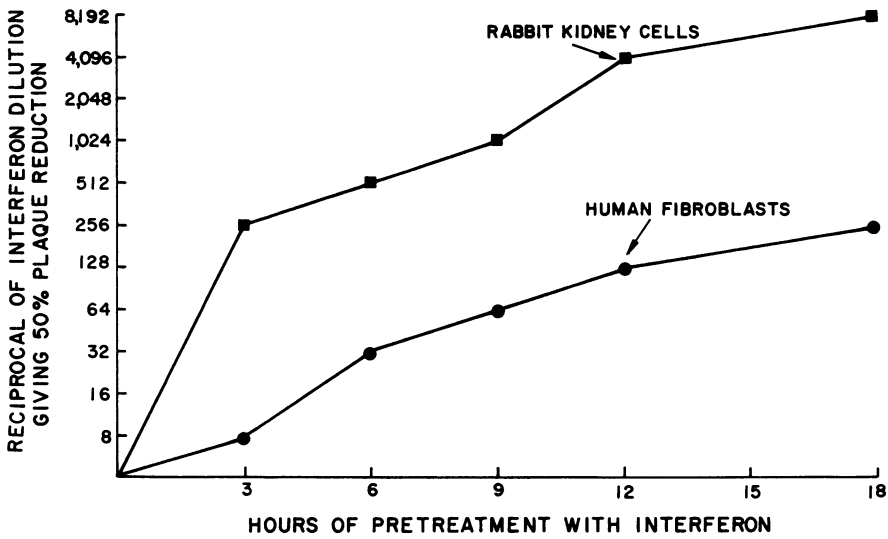

FIG. 2.—Time response of human foreskin fibroblasts (●) and rabbit kidney cells (■) to stock interferon, obtained by NDV infection of human foreskin fibroblasts, and assayed by VSV plaque reduction.

active principle in rabbit cells might be different from the active principle in human cells.

The effect of different periods of pretreatment of human and rabbit cells was compared. Maximum effect of both human interferon and ISF activities was sometimes reached within 6 hours, but on other occasions up to 18 hours of pretreatment were needed before maximum interference was achieved. No increase in activity was noticed after 24 hours. Figure 2 illustrates the kinetics of ISF and human interferon activities in experiments where a lengthy exposure was required for complete effect. The limit of activity appears to be attained asymptotically in both cases, following parallel curves for the values measured. A parallel, 32-fold increase in interference is found between 3 and 18 hours in both cell types. In these and other experiments, the difference in titer found between ISF and human interferon appears to be accounted for by an early build-up of interference in rabbit cells, which is not demonstrable in human cells.

The interferon preparation was active in human cells against rubella virus and poliovirus, as measured by virus replication in tube monolayers; against VSV and Sindbis virus, as measured by plaque reduction; and against NDV, as measured by hemadsorption. In order to ascertain that the inhibitory action of ISF was not limited to VSV, its effect on other viruses in rabbit kidney cells was investigated. Monolayer cultures of about 150,000 cells in tubes were treated overnight with stock interferon at a dilution of 1/100, or approximately 50 units in 1 ml of maintenance medium. Cells were then washed, and 300 PFU of mengovirus were added and allowed to adsorb for one hour. After appropriate incubation in 1 ml of maintenance medium, six replicate tubes were harvested, frozen and thawed twice, pooled, and titrated. Untreated cultures yielded 2×10^5 PFU/ml after 24 hours of incubation, and 3×10^5 PFU/ml after 48 hours. In cultures treated with ISF, the total harvest was reduced by 99 per cent after 24 hours, and

by 95 per cent after 48 hours of incubation. Thus mengovirus, a virus sensitive to several varieties of rabbit interferon in rabbit cells,[8] was also sensitive to ISF. The effect of ISF on rubella virus multiplication in rabbit cells was examined under the same conditions as for mengovirus, except that an input of 1×10^4 PFU was used. The cells were washed four times after adsorption. After 24 hours, control yields in two experiments were 1 and 4×10^4 PFU/ml, respectively. In interferon-treated cultures, there was a reduction of 80 and 90 per cent after 24 hours; after this, the inhibitory effect rapidly declined. It appears that ISF is active on rubella virus, although less than on VSV (see Table 2). For comparison, rabbit interferon is active on rubella virus in rabbit cells,[9] but apparently less so than on VSV.[10] Vaccinia virus, when tested for plaque reduction in rabbit kidney cells by the same method as used for VSV, was insensitive to rabbit serum interferon (titer 1/256 against VSV). In similar fashion, ISF also was inactive against vaccinia virus. It is concluded that ISF, like interferon, is active against unrelated viruses and may fail to be active on a given virus under given conditions; its activity on unrelated viruses in rabbit cells parallels that of homologous interferon.

In order to establish whether the activity of ISF, like that of interferon, depends on formation of host messenger RNA, the effect of pretreatment with dactinomycin on the inhibitory power of ISF was investigated. This was ascertained by measuring the virus yield as well as inhibition of VSV plaque formation in rabbit cells appropriately treated with dactinomycin and stock interferon. Treated and control tube cultures were inoculated with 300 PFU of VSV and harvested after 36 hours of incubation. The results of a typical experiment are shown in Table 2. It is seen that ISF inhibits the replication of VSV, as well as plaque formation, in rabbit cells; that appropriate amounts of dactinomycin enhance VSV replication in these cells, and that the effect of ISF is suppressed by pretreatment with the drug. Treated and control cultures in Petri dishes were observed for plaque formation after four days. The dactinomycin concentration used, 0.2 μg/ml, was critical with regard to optimal activity and lack of toxicity. As shown in Table 3, when virus was added within six hours after the dactinomycin pulse, the effect of ISF was completely deleted (expts. 1 and 2). In conclusion, ISF is similar to interferon in that its action is prevented by dactinomycin.

Search for NDV components: Noninfectious NDV particles may render cells refractory to superinfection with virulent virus, and make them release interferon which protects the remaining cells.[11, 12] The hypothesis was considered that the stock interferon preparation might contain particles which, either through primary interference or by production of "secondary" rabbit interferon, could account for ISF activity. Although consideration of acid treatment, centrifugation, dose response, and dactinomycin response[13] made such an explanation unlikely, evidence was sought for the presence of active NDV components in the stock interferon preparation.

Stock interferon was found to be free of live NDV. No cytopathic effect was observed when chick embryo fibroblasts were inoculated with different dilutions. The preparation did not kill 11 day chick embryos when administered in the chorioallantoic sac. Hemagglutinin was not detectable in the allantoic fluid three days later, nor in stock interferon. An attempt was made to neutralize

TABLE 2. *Growth of VSV in rabbit kidney cells treated with dactinomycin and/or ISF.*

	Dactinomycin Dosage			
	(5 μg/ml)	(1 μg/ml)	(0.2 μg/ml)	None
ISF dosage: 50 units*	7†	50	40	1
12 units	5	60	20	2
3 units	5	90	60	9
None	5	100	200	30

* PR_{50}-units (see text) per 1 ml per tube; 6 hr pretreatment.
† PFU × 10^4/ml. Input VSV: 300 PFU/150,000 cells; 36-hr growth in 1 ml. Pooled total harvest of 5 replicate tubes.

TABLE 3. *Plaque formation of VSV in rabbit kidney cells treated with dactinomycin and/or ISF.**

	Dactinomycin		No Dactinomycin	
Expts.	ISF	No ISF	ISF	No ISF
1	96	105	16	100
2	57	55	3	100
3	14	53	0	100
4	30	80	2	100

* Dactinomycin, 0.2 μg/ml; ISF, 50 PR_{50}-units.
Pretreatment with ISF: expts. 1 and 2, 6 hr; expts. 3 and 4, 18 hr.
Numbers refer to percentage of plaques as compared to untreated controls.

hypothetical particles with NDV antiserum, thus aborting their interfering capacity. This might reduce ISF activity while leaving the human interferon activity intact. Stock interferon was mixed with an equal volume of rabbit NDV-antiserum, incubated for one hour at 37°, and assayed for VSV plaque-reducing activity on foreskin and rabbit kidney cells. A 1/1250 dilution of this antiserum, after one hour of contact at 37°, reduced NDV infectivity by 90 per cent, as compared to a control mixture without antiserum. Both antiserum-treated and control interferon showed titers of 1/128 on human cells, and 1/4096 on rabbit cells. Thus, ISF activity was not reduced by NDV antiserum.

In light of the above, the early increase of interference in rabbit cells could be accounted for by primary interference, while the subsequent release of rabbit interferon might contribute to the further progressive increase in the protected cells. Two ml of undiluted stock interferon were incubated for three hours on rabbit kidney monolayers in Petri dishes. The cells were then thoroughly washed, and maintenance medium added. Media were collected after 12 and 24 hours, and assayed for VSV plaque-reducing activity on rabbit kidney cells, starting with undiluted medium. No rabbit interferon activity was detected. In another type of experiment, 2 ml of stock interferon diluted fivefold were added to rabbit kidney and to human foreskin monolayers in 16-oz prescription bottles. After two hours, 30 ml of serum-free medium were added, and the cells were further incubated as described under *Materials and Methods* for the production of interferon. Samples of the fluid from the rabbit kidney cultures were taken immediately after addition of the medium, and after 48 hours of incubation. Their ISF activity, corrected for dilution, was 1/5120 in both cases. The fluids from the foreskin cultures had an ISF activity of 1/2560 even after incubation. Wide variations in recovery of interferon activity are expected if the ISF effect is due

TABLE 4. *Characterization of antiviral activity of a human interferon in rabbit cells (interspecific factor) and in human cells (human interferon).*

Properties investigated	Interspecific factor	Human interferon	Data on interferons from literature
Acid-stable (pH 2)	+	+	+
Heat-resistant	+	+	+
Nondialyzable	+	+	+
Nonsedimentable (at 100,000 *g*)	+	+	+
Trypsin-sensitive	+	+	+
Resists antibody to inducer virus	+	+	+
Does not inactivate virus	+	+	+
Does not induce interferon production	+	NT*	+
Activity not eluted by washing cells	+	+	+
Lack of significant uptake by cells	+	NT	+
Maximal effect requires hours	+	+	+
Typical linear dose response	+	+	+
Active on unrelated viruses	+	+	+
Certain viruses insensitive	+	NT	+
Activity prevented by dactinomycin	+	NT	+

* NT: not tested.

to particles which adsorb to cells to give rise to interference through primary interference or through secondary interferon production in the rabbit cells. No such variations were observed, indicating again that such particles play no role in ISF activity. In the absence of production of "secondary" interferon, the recovery of ISF in amounts equal to the input clearly requires thermostability of ISF, as has been shown above, and lack of significant uptake or degradation by cells, a property demonstrated for known interferons in certain cell systems.[14]

Discussion.—Interferon is the only substance known which has the properties of the interspecific factor (ISF) described in this study. These are summarized in Table 4. There are no indications that ISF consists of several inhibitors with different properties; if this were so, a discrepancy with some of the properties of interferon listed would most likely have been uncovered. At this stage, it is not possible to say whether ISF is coded for by the host genome, or whether it is inducible by a large number of viruses, as is the case for known interferons. The range of cells capable of producing interspecific interferons and the range of susceptible target cells remain to be determined.

The finding that ISF is active at higher titer in rabbit kidney cells does not imply that it is different from the interferon which is active in human fibroblasts. Different cells, or viruses in different hosts, may have a different sensitivity to interferon. There is little doubt that the foreskin fibroblastic cells used in this study were healthy human cells with normal sensitivity to interferon, since certain human interferons were active in the foreskin cells and not in rabbit cells. Also, measles-induced serum interferon[1] when tested in human foreskin cells gave titers which were comparable with those reported by other authors.[15]

The finding that partially purified chick interferon was no longer active on mouse cells, and vice versa, has led to the generalization that cross-reactivity of interferons is due to impurities, at least among widely separated species.[16, 17] However, the abundance of findings of heterologous activity of interferons (re-

viewed by Lockart[18]) is in singular contrast with the paucity of studies aimed at determining the nature of the inhibitor for heterologous cells. There is one mention of challenge of interferon-treated heterologous cells with unrelated viruses,[19] and no mention of the effect of dactinomycin on the heterologous activity of interferons—a criterion considered essential for the acceptance of an unknown inhibitor as an interferon.[18]

Our result with the measles-induced interferon is similar to that of Michaels *et al.*[20] and Merigan *et al.*[3] who found that human serum interferon is not active on rabbit cells. This implies that human interferons fall into two classes: one active in human cells and not in rabbit cells, the other active in rabbit cells or in both human and rabbit cells. On the basis of physicochemical differences, several molecular varieties of interferon are discernible within the same species. The present findings provide a biological marker for different interferons produced by a single species.

Summary.—Interferon prepared under defined conditions in human foreskin cells is 20 times more active against vesicular stomatitis virus in rabbit cells than in human cells. The properties of the inhibitor for heterologous cells were found to be identical with those of interferon. The phenomenon appears to provide a biological marker for different interferons produced by human cells.

Note added in proof: Since this paper was submitted, Dr. T. C. Merigan (Stanford University School of Medicine) has informed us that he has confirmed the activity on rabbit cells of human interferon prepared as described, and that he has been unable to separate the activity of human interferon in human and in rabbit cells by differential precipitation and by chromatography.

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