Hellems, H. K., Circulation Res. 11, 17 (1962).

15. Ho, R. J., Jeanrenaud, B., Posternak, T. H., Renold, A. E., Biochim. Biophys. Acta 144, 74 (1967).

16. Butcher, R. W., Sneyd, J. G. T., Park, C. R., and Sutherland, E. W., Jr., J. Biol. Chem. 241, 1651 (1966).

Received Nov. 27, 1967. P.S.E.B.M., 1968, Vol. 127.

Induction of the Interferon System by Various Inducers*† (32924)

CHARLES E. BUCKLER, KEN T. WONG,[1] AND SAMUEL BARON

U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Biology of Viruses, Bethesda, Maryland 20014

Several laboratories (1,2) have shown that the development of interferon induced antiviral activity can be blocked by Dactinomycin (actinomycin D) and concluded that DNA-directed RNA synthesis was involved in this system. These findings also suggest that interferon does not itself inhibit virus replication but that it stimulates the production of new macromolecules by the cell—the proposed antiviral substance(s) (AVS) (1). The present report describes a comparative study of the interferon response and the development of resistance (proposed AVS?) in primary mouse embryo (ME) and African green monkey kidney (AGMK) cell cultures treated with virus, statolon, or interferon. The results indicate that a major portion of virus-induced interference in AGMK could be attributed to the interferon system, and that cellular resistance in ME and AGMK frequently develops before detectable interferon production.

Materials and Methods. The detailed procedures in this study were similar with those described in previous reports (3,4).

Inducers. The PR8 strain of influenza A virus, Semliki Forest virus (SLFV), rubella virus, Sindbis virus, monkey interferon or statolon (kindly supplied by Dr. W. Kleinschmidt, Eli Lilly Co.) (5) were used for induction of the interferon system in AGMK cell cultures. Statolon, polyoma, SV40, Sendai, and Newcastle disease (ND) viruses were employed in ME cell cultures.

Assay of interferon. The acidification method for destroying residual infectious virus in interferon preparations was previously described (3). Interferon was assayed in appropriate cell cultures by inhibition of vesicular stomatitis virus (VSV) yield. A unit of interferon was determined as the highest dilution of the sample which inhibited the final yield of VSV by 0.5 $\log_{10}$ during a single growth cycle (4). The titer of the NIH reference mouse interferon is 500 units/ml by this method. The AVS was estimated indirectly by determining the degree of cellular resistance to multiplication of VSV. Significant resistance was determined to begin at 0.5 $\log_{10}$ reduction of the final yield of VSV during a single growth cycle (6).

Assay of challenge viruses. All challenge viruses (SLFV, VSV, poliovirus type I - Mahoney strain, Sindbis virus or rubella virus) used in this experiment had at least a titer of $10^{7.0}$ plaque forming units (pfu) or 50% rubella interference doses (InD_{50}) per ml in order to insure a virus to cell multiplicity of greater than 10 for a single growth cycle infection. The yield of infectious virus harvested from the interference test was determined by plaque or infectivity titrations after treatment with the appropriate antiserum as described elsewhere (3,4,7).

Results. Dynamics of interferon response in

* Parts of these studies were conducted in the Department of Virus Research, Microbiological Associates, Inc., Bethesda, Maryland.

† Some aspects of this study were presented at the 2nd International Symposium for Medical and Applied Virology, Fort Lauderdale, Florida, Nov., 1966, and the International Symposium on Interferons, Siena, Italy, June 1967.

[1] Present address: Division of Infectious Diseases, Department of Medicine, Stanford University School of Medicine, Palo Alto, California.

ME cell cultures treated with various inducers. Groups of 3 ME tube cultures were stimulated with statolon, interferon, or virus and then the development of cellular resistance to VSV and the production of interferon was followed. Tubes treated with statolon or virus were washed after 2 hours at 37°C to remove unadsorbed inducers. Figure 1 shows the development of such resistance in ME cells. Resistance was induced by NDV, statolon, or interferon, whereas no resistance developed following infection by polyoma, SV40, or Sendai viruses. The small dose of interferon (10 units/ml) induced resistance somewhat more slowly and to a lesser degree than did the large doses of NDV, or statolon. In subsequent experiments a larger dose of interferon (1,000 units/ml) induced resistance by 1.5 hours which is 2.5 hours before induction of resistance by large doses of NDV or of statolon.

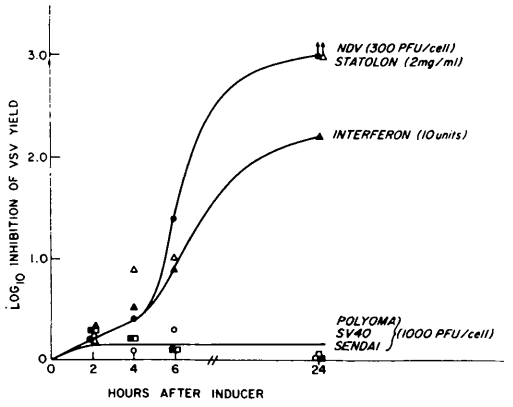

FIG. 1. Induction of cellular resistance to VSV in ME cell cultures treated with various inducers.

The production of interferon was determined by assaying the fluids from these induced cultures. No detectable interferon (less than 2 units/ml) was produced by the NDV or statolon treated ME cells through 8 hours but was detectable by 14–16 hours. The maximal amount of interferon (100 units/ml) was produced by 24 hours. These findings indicate that detectable interferon production can occur subsequent to the production of AVS and the significance will be discussed later.

Decline of cellular resistance to virus after removal of inducer. To estimate the rate of decline of cellular resistance following removal of the various inducers, ME cultures were first treated with doses of interferon, NDV, or statolon sufficient to inhibit VSV yield more than 3 $\log_{10}$. The cultures were carefully washed at 24 hours and again at 48 hours to remove the interferon which was applied or which was induced by 24 hours after treatment with NDV or statolon. Figure 2 shows

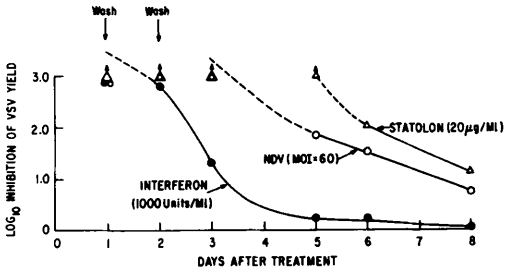

FIG. 2. Decline of cellular resistance to VSV in ME cell cultures after removal of inducers.

the rate of loss of resistance for each of the inducers when resistance declined through the measurable region below 3 $\log_{10}$ inhibition of VSV yield. It may be seen that once resistance began to decline there was a more rapid loss of the resistance induced by interferon than that induced by NDV or statolon. It has been previously demonstrated that induction of AVS by interferon is rapidly stopped after simple removal of interferon (8).

Induction of cellular resistance and production of interferon in AGMK cells induced by several viral and nonviral stimuli. Experiments similar to those in ME cells were conducted in AGMK cells and are summarized in Fig. 3. Specifically 6 groups of AGMK tube cultures were treated with virus, interferon, or statolon for 2 hours and then the development of cellular resistance to VSV and production of interferon was examined. As shown in Fig. 3, cellular resistance was induced following application of the various stimuli, as evidenced by the inhibition of multiplication of VSV. The relatively low level of resistance induced by interferon is attributable to the removal of interferon after only 2 hours of treatment. The delayed development of resistance induced by statolon is attributable to the relatively low doses used since larger doses of statolon resulted in earlier and greater resistance (Table II). The results are similar to

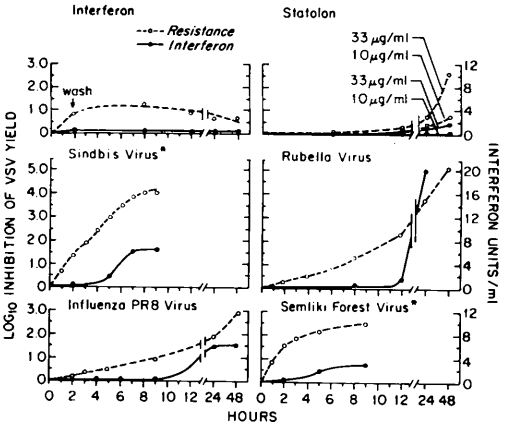

FIG. 3. Comparative induction of interferon and resistance to VSV in AGMK cell cultures treated with various stimuli. Dose levels: interferon, 32 units; virus input, 10-20 pfu or InD_{50} of virus cell. *CPE was observed 12 hours after virus infection.

those obtained in ME cells in that resistance was again generally detectable before interferon production.

Characterization of antiviral activity in AGMK cells induced by various inducers. For characterization of resistance, AGMK culture tubes were treated with the inducers listed in Table I. After two hours, unadsorbed inducer was removed by washing and the cultures were refed. They were then incubated until times of virus challenge and times of harvest of fluids for assay of interferon. As may be seen, the resistance to challenge virus multiplication induced by Sindbis virus, rubella virus, interferon, and statolon, extended to a broad range of viruses and inhibited the interferon-sensitive viruses to a greater degree than the relatively interferon-resistant poliovirus. In addition cultures treated with statolon or the various viruses eventually produced interferon. These characteristics of the statolon and virus-induced resistance are similar to those of the interferon system (9).

Another property shared by the resistance induced by various stimuli (Table II) is its prevention by prior treatment with Dactinomycin, indicating a requirement for continued host-cell RNA synthesis. As shown in Table II, pretreatment of AGMK cell cultures with various inducers plus enough antibiotic (0.5 ml of 64 μg/ml) to inhibit 90% of cellular RNA synthesis (10), severely inhibited the induction of cellular resistance to VSV and poliovirus. When the dose was increased to 200 μg/ml of Dactinomycin, in subsequent experiments, the small degree of residual resistance shown in Table II was virtually abolished. This finding agrees with the previous report (1) that a property of interferon-induced resistance is its inhibition by Dac-

TABLE I. Induction of Interferon and Cellular Resistance to Challenge Viruses in AGMK Cells Treated with Various Inducers.

Inducers	Time of challenge	Interferon yield (units/ml)	Reduction of virus yield (log_{10} pfu)				
			VSV	Sindbis	SLFV	Polio I	Rubella[a]
Interferon[b]	8	<1.0	1.2	1.3	1.0	0.2	—
(3 units/ml)	24	<1.0	0.9	1.1	1.1	0.6	1.0
Statolon (10 μg/ml)	48	<1.0	0.5	0.6	0.4	0.0	—
(33 μg/ml)	24	<1.0	0.8	—	—	—	—
	48	3.0	2.6	—	—	—	—
Rubella virus	8	<1.0	1.4	0.3	0.8	0.1	—
(MOI 10:1)[c]	24	20.0	3.9	3.0	3.0	2.0	—
Sindbis virus	2	<1.0	1.4	—	0.9	0.0	—
(MOI 12:1)	5	2.0	2.9	—	3.1	0.5	—
	7	6.0	3.8	—	3.9	0.5	—

[a] Titer in 50% interference doses (InD_{50}) against Echo-11 challenge virus.

[b] Monkey interferon induced by Kunz influenza virus which was applied onto AGMK cells for 2 hours only and then washed before the fresh maintenance medium was added.

[c] MOI indicates multiplicity of infection.

TABLE II. Effect of Dactinomycin on Induction of Interferon and Resistance in AGMK Cell Cultures Treated with Various Inducers.

Inducers	Hours after treatment	Interferon yield (units/ml)		Reduction of virus yield (log_{10} pfu)			
				VSV		Polio I	
		None	Dact.[a]	None	Dact.	None	Dact.
Interferon (32 units/ml)	12	<1.0	<1.0	1.4	0.7	—	—
Sindbis virus (MOI 12:1)	4	<1.0	<1.0	1.6	0.7	0.6	0.3
	8	8.0	<1.0	3.7	2.4	1.1	0.3
Rubella virus (MOI 10:1)	20	14.0	2.0	—	—	2.0	0.4
	24	—	—	4.7	0.7	3.2	0.5
Statolon (200 μg/ml)	24	3.0	<1.0	1.6	0.3	—	—

[a] Dactinomycin (64 μg/ml) treatment of cultures at time of application of inducer.

tinomycin. The inhibition by Dactinomycin of the development of resistance eliminates the possibility that the resistance is due to a general toxic effect of the virus on the cells. As in other systems (11) the resistance induced by virus and interferon has four properties associated with the interferon system (a) nonspecific inhibition of virus, (b) lower sensitivity of poliovirus to the antiviral effect, (c) inhibition by Dactinomycin of induction of resistance, and (d) eventual production of detectable interferon by the resistant cells.

Discussion. Cellular resistance to viral infection (AVS) consistently appeared before detectable interferon in ME and AGMK cell cultures stimulated with statolon or diverse viruses. Results simular to those described in this report have also been obtained in a West Nile virus–mouse cell system. In these experiments it was found that West Nile virus infection induced high levels of resistance in cell cultures derived from two different strains of mice 18–24 hours prior to the time when the first detectable interferon was produced.[2] Others have previously observed detectable resistance before detectable interferon (12) but under the experimental conditions used it could not be excluded that the time of AVS production was actually much later than the time of virus challenge for determination of resistance. Theoretically, AVS could be produced late during the challenge virus growth cycle and still inhibit the formation of late viral messenger RNA (mRNA). Thus it could not be excluded that resistance actually began after first detectable production of interferon. This possibility has been excluded for the present experimental conditions in ME cells by Dianzani *et al.* (8). They showed that AVS is rapidly translated from its mRNA by the lack of increase of resistance after addition of Dactinomycin to interferon treated cultures. The development of resistance, with time, after interferon treatment was then shown to be unaffected by the addition of Dactinomycin at the time of VSV challenge, which indicated rapid translation. The conclusion from these observations is that the time of VSV challenge as used in determining the development of resistance is very close to the time of AVS production.

Although the present findings imply that virus infection may induce AVS independently of induction of interferon, an alternate explanation seems equally likely. It remains possible that subdetectable amounts of intracellular interferon are first induced and this intracellular interferon induces the AVS. Consistent with this interpretation is the observation that when interferon induces resistance in cell culures a subdetectable amount is consumed (13). Also consistent is the finding that a large dose of interferon can induce AVS in ME cells more rapidly than do large doses of NDV or statolon. Perhaps the longer induction times by NDV or statolon are due to

[2] Hansen, B., Koprowski, H., Buckler, C. E., and Baron, S., personal communication.

a requirement for induction of intracellular interferon as a necessary precursor to induction of AVS.

Characterization of the interference induced in AGMK cells by statolon, Sindbis virus and rubella virus indicates similarity to interferon-induced resistance (8). Specifically the resistances were (a) nonspecific for challenge virus, (b) more effective against challenge viruses of greater sensitivity to interferon than against the more interferon-resistant poliovirus, (c) prevented by Dactinomycin, and (d) eventually accompanied by production of detectable interferon. Although the present forms of interference or cellular resistance can be largely attributable to the interferon system, other forms of interference could have been occurring independently (14–16). To determine what fraction of the many instances of viral interference is mediated by the interferon system, it will be necessary to characterize many such interference phenomena.

The inability of certain virus infections to induce resistance (Fig. 1) has not been studied further to determine whether the interferon system is not stimulated or whether there is active suppression of the interferon system. It is of interest that the two tumor producing viruses studied failed to induce resistance in ME cells although they are known to initiate infection. This observation has not been pursued. Under experimental conditions polyoma virus may induce the interferon system in mouse cells (17,18).

There are several possible interpretations for the less rapid loss of resistance in cells treated with NDV and statolon. We favor the interpretation that after washing, NDV and satolon, unlike interferon, continue to stimulate the cells in a decreasing degree from an intracellular site.

Summary. Studies of the comparative induction of interference by virus, statolon, and interferon in mouse embryo (ME) and African green monkey kidney (AGMK) cell cultures were performed. The major portion of the interference induced by different viruses in AGMK was characterized as being interferon-like. In both cell systems resistance was observed to develop before appearance of detectable interferon. Decline of resistance after removal from ME cells of unadsorbed statolon and NDV was slower than after removal of interferon. Possible significances of these observations are considered.

1. Taylor, J., Biochem. Biophys. Res. Commun. **14**, 447 (1964).

2. Lockart, R. Z., Biochem. Biophys. Res. Commun. **15**, 513 (1964).

3. Wong, K. T., Baron, S., and Ward, T. G., J. Immunol. **99**, 1140 (1967).

4. Baron, S., Buckler, C. E., Levy, H. B., and Friedman, R. M., Proc. Soc. Exptl. Biol. Med. **125**, 1320 (1967).

5. Kleinschmidt, W. J. and Probst, G. W., Antibiot. Chemotherapy **12**, 298 (1962).

6. Baron, S., Buckler, C. E., Levy, H. B., and Friedman, R. M., Proc. Soc. Exptl. Biol. Med. **125**, 1320 (1967).

7. Parkman, P. D., Arch. Ges. Virusforsch. **16**, 403 (1965).

8. Dianzani, F., Buckler, C. E. and Baron, S., Proc. Intern. Symp. Interferons, Siena, Italy, June, 1967, in press.

9. Isaacs, I., Advan. Virus Res. **10**, 1 (1953).

10. Wong, K. T., Baron, S., Levy, H. B., and Ward, T. G., Proc. Soc. Exptl. Biol. Med. **125**, 65 (1967).

11. Friedman, R. M., Nature **201**, 838 (1964).

12. Henderson, J. R. and Taylor, R. M., Virology **13**, 477 (1961).

13. Buckler, C. E., Baron, S., and Levy, H. B., Science **152**, 80 (1966).

14. Henle, W., J. Immunol. **64**, 203 (1950).

15. Schlesinger, R. W., in "The Viruses; Biochemical, Biological, and Biophysical Properties," Burnet, F. M., and Stanley, W. M., eds., Vol. 3, p. 157. Academic Press, New York, 1959.

16. Marcus, P. I. and Carver, D. H., J. Virol. **1**, 334 (1967).

17. Friedman, R. M., Rabson, A. S., and Kirkham, W. R., Proc. Soc. Exptl. Biol. Med. **112**, 237 (1963).

18. Glasgow, L. A. and Habel, K., Virology **19**, 328 (1963).

Received Nov. 27, 1967. P.S.E.B.M., 1968, Vol. 127.