*AN INHIBITOR OF VIRAL ACTIVITY APPEARING IN INFECTED CELL CULTURES**

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Introduction.—Cytopathogenic viruses may lose their cell-destroying capacity under centain conditions. For example, Type II MEF_1 poliovirus adapted to chick embryo (RMC virus)[1] fails to induce manifest cytopathogenic effect (CPE) in human kidney tissue cells, and multiplies but slightly. In contrast, its capacity under certain conditions to multiply in and destroy human amnion cells is retained.[2, 3] At the outset of this investigation it was noted that CPE of RMC virus in fluids from infected human kidney and amnion cell cultures, in contrast to virus from chick embryos, was markedly inhibited when low dilutions of such fluids were employed as inocula. When the same fluids were further diluted the virus exerted the usual destructive effect on amnion cells. It was thereafter determined that such fluids from human kidney cultures not only inhibited the effect of RMC virus but also certain other viral agents. Experiments here summarized were then undertaken to elucidate this phenomenon.

Materials and Methods.—A stock of RMC virus from the 143rd chick embryo passage and stock strains maintained in this laboratory of polioviruses Type I (Brunhilde) and Type III (Leon), herpes simplex, Sindbis, and vaccinia viruses were employed.

Monolayer cultures were established in test tubes (140 × 15 mm) or Roux bottles with of trypsinized human renal cells which were prepared by Bodian's method[4] and suspended in bovine amniotic fluid medium with 5 per cent normal horse serum (BAF medium).[5] Aliquots of 1 and 50 ml of suspension (3×10^5 cells/ml) were added respectively to tubes and bottles. Cultures were maintained with BAF medium and used experimentally approximately 2 weeks after they were initiated.

For titration and detection of RMC virus, primary cultures of trypsinized human amnion cells prepared according to procedures previously described[6] and nourished with BAF medium were used 2 weeks or longer after initiation and rolled after inoculation. Under these conditions, this virus produces marked CPE. To test the effect on other viruses of "virus-inhibitory fluid" (VIF) containing infective RMC virus, amnion cell cultures about 2 weeks old were left stationary after inoculation. Under these circumstances, RMC virus is not cytopathogenic.[3] In consequence, observed CPE could be confidently attributed to the other virus. All cultures were incubated at 37°C.

VIF was prepared in Roux bottle cultures of human kidney cells containing 50 ml of medium. Approximately 10^5 TCD_{50} RMC virus were added to each bottle. After one day the medium was changed, replaced, and harvested as VIF on the third day. As control for the activity of VIF, fluid was harvested from uninoculated kidney cell cultures prepared with aliquots of the same cell suspension and

thereafter treated in the same manner. This material is referred to as "CF" (control fluid).

Results.—When VIF is titrated in rolled amnion cell cultures moderate to small quantities of RMC virus are demonstrable (Table 1). CPE of this virus in undiluted VIF or in low dilutions of the fluid is, however, completely or almost completely suppressed. To determine whether this inhibitory effect was dependent upon the presence of infective virus or viral antigens and whether this effect was specific in respect to RMC virus or extended to other agents, two types of experiments were done. In the first, VIF was centrifuged at high speed and the supernatant fluid tested for inhibitory effect against RMC virus as well as against polioviruses Types I and III. In the second series of experiments Type II poliovirus antiserum[7] was added in sufficient quantity to VIF to neutralize the virus. The mixture was then tested for inhibitory properties against a number of other viruses.

TABLE I

CPE OF FRACTIONS OF VIF

Dilution of Fraction	Fraction of VIF			Supernatant§ + RMC virus
	Uncentrifuged	Supernatant†	Sediment‡	
Undiluted	0*	0	++++	±
	±	0	++++	
1:2	N.D.	N.D.	N.D.	±
1:4	N.D.	N.D.	N.D.	0
1:8	N.D.	N.D.	N.D.	±
1:16	N.D.	N.D.	N.D.	+
$1:10^1$	++	0	++++	
	++	0	++++	
$1:10^2$	++++	0	++++	
	++++	0	++++	
$1:10^3$	++++	0	0	
	0	0		
$1:10^4$	0	0	0	

0 = no cell destruction.
± = questionable cell destruction.
+ = estimated 25% cell destruction.
++ = estimated 50% cell destruction.
+++ = estimated 75% cell destruction.
++++ = estimated 100% cell destruction.
* = duplicate cultures; 0.1 ml of each fraction was added to a culture. Cultures were observed for 2 weeks after inoculation.
N.D. = not done.
† = obtained by centrifugation in Spinco No. 40 rotor at 104,500 g's for 90 min.
‡ = sediment includes bottom 0.5 ml after centrifugation and button.
§ = amnion cell cultures were overlaid with dilutions of supernatant and each inoculated with 10^3 TCD_{50} stock RMC virus. Cultures overlaid with CF gave ++++ CPE.

From the data in Table 1 it is evident that centrifugation removed all detectable infective RMC virus from VIF. When the virus-free supernatant fluid was added to amnion cell cultures it inhibited CPE of freshly added RMC virus. Furthermore, as shown in Figure 1, CPE of poliovirus Type I was also markedly depressed by the same supernatant fluid. Similar results were obtained with poliovirus Type III.

To 0.8-ml aliquots of VIF, 0.2-ml dilutions of poliovirus Type II monkey antiserum increasing by a factor of 2 were added to a final concentration ranging from 1:5–1:320. A neutralizing antibody titer of 1:360 against 100 TCD_{50} stock RMC virus was found previously for this antiserum. The mixtures were kept at 4°C for 24 hr, and then added to amnion cell cultures after removal of the medium. As controls, a comparable series of mixtures were made employing CF in place of

VIF. After addition of 10^3 TCD_{50} poliovirus Type I to each culture, they were incubated for 8 days. In all controls CPE was minimal at the end of 3 days and maximal after 8 days. In all cultures containing VIF, CPE was absent on the third day and minimal on the eighth day. Similarly VIF with and without antiserum inhibited the action of Types I and III poliovirus in human kidney cell cultures, as well as CPE of Sindbis, vaccinia, and herpes simplex viruses in human amnion or kidney cell cultures in preliminary studies.

Further evidence in support of these observations was obtained by exposing amnion cell cultures to a concentration of stock RMC virus equivalent to that found in VIF. Three hours later, 10^2–10^3 TCD_{50} Type III poliovirus were added and the cultures incubated. The characteristic CPE of Type III virus was not affected, indicating that the presence of such a dose of RMC virus *per se* does not interfere with the course of infection by Type III virus.

Taken as a whole, these results indicate that the inhibitory property of VIF is not dependent upon the presence of infective virus or virus materials that under the experimental conditions can be removed by centrifugation or inactivated by specific antiserum. They also show that the inhibitory effect extends to heterologous strains of poliovirus as well as to other unrelated agents.

Preliminary studies on the mode of action of the inhibitory factor have been carried out. These were aimed at determining whether VIF (*a*) acted directly on the virus, (*b*) decreased adsorption of virus to cells, (*c*) diminished viral multiplication.

(*a*) To aliquots of VIF and CF sufficient poliovirus Type II antiserum was added to yield a final concentration of 1:50. To portions of each mixture an equal volume of poliovirus Type I was added (10^6 TCD_{50}/0.1 ml). After standing at 4°C for 24 hr, dilutions of each mixture were prepared and titrated in amnion cell cultures employing a dilution factor of 3.16 and 3 tubes per dilution. The titers found did not differ significantly. Accordingly VIF does not under these conditions inactivate the virus.

(*b*) Monolayer cultures each containing about 10^5 to 10^6 human kidney cells were exposed either to a mixture of VIF and poliovirus Type II antiserum (final concentration 1:50) or to a mixture of CF and antiserum. The cultures were rolled for 3 hr at 37°C when about 10^6 TCD_{50} poliovirus Type I were added to each. After 1 hr at 37°C the cells were washed five times, treated with poliovirus Type I antiserum, removed from the glass with 0.05 per cent trypsin and counted. Dilutions of the cell suspension were prepared (dilution factor 3.16) and aliquots of each dilution added to amnion cell cultures. The results of the titrations indicated that in both preparations about 1 of 8 cells had absorbed virus. Similar results were obtained using the virus-free supernatant of VIF, without the addition of antiserum. These results, therefore, indicate that VIF does not interfere with adsorption of virus to the cells under the circumstances described.

(*c*) To each of one set of 10 cultures of amnion cells 1 ml VIF freed of detectable RMC virus by centrifugation and to another set 1 ml CF were added after removal of the medium. After standing for 3 hr at 37°C 10^3 TCD_{50} Type I poliovirus were added and the cultures incubated. At the intervals shown in Figure 1 the degree of CPE was recorded and one culture from each set was removed and stored at −20°C. At the end of the fourteenth day the cultures were frozen and thawed

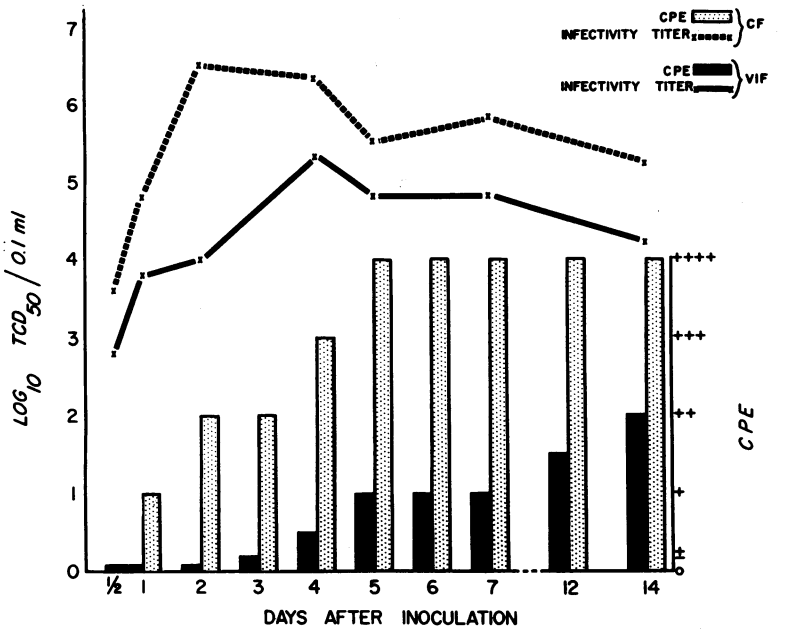

FIG. 1.—Effect of VIF on growth and CPE of Brunhilde Type I poliovirus in human amnion cell cultures. The VIF used was the supernatant fraction which was shown to be free of detectable virus (see Table 1).

three times employing an alcohol-dry ice mixture. The suspensions were then centrifuged at 2,000 rpm for 10 min and the supernatant fluids assayed for infectivity in amnion cell cultures. The efficiency of the release of intracellular virus by freezing and thawing was demonstrated in separate experiments. The results summarized in Figure 1 show that the total yield of virus was consistently less in cultures exposed to VIF in which CPE was likewise markedly diminished. Since the experiments were designed to measure the total virus in the two systems at various intervals the findings suggest that in the presence of VIF viral synthesis is reduced. These conclusions were confirmed in another experiment where extracellular and intracellular virus were measured independently.

The data recorded in Figure 1 also suggest that VIF may serve to convert a "virulent" infection characterized by complete destruction of the cell population to a chronic one in which cells continue to survive or multiply and virus to propagate. Additional evidence that VIF may play a role of this sort is provided by experiments in which fluid from HeLa cell cultures chronically infected with RMC virus for over 1 year was found effective in inhibiting CPE of polioviruses Types I and III and Sindbis virus in amnion cell cultures.

Activity of VIF was not removed by dialysis at 4°C against Hanks' solution and BAF medium. It was reduced but not destroyed by heating at 56°C for 30 min, and remained unchanged after repeated (four times) freezing (dry ice bath) and thawing (37°C), and after storage for 1 month at 4°C. Occasionally undiluted CF exhibited a partial inhibitory effect on polioviruses Types I and III that was slightly enhanced in the presence of Type II poliovirus antiserum. Unlike that of VIF the effect of CF was completely eliminated on slight dilution.

Comment.—A factor inhibiting CPE and probably multiplication of several viral strains has been demonstrated in culture fluids of human renal cells infected with a chick embryo adapted strain of Type II poliovirus. The inhibitor can be separated from infective virus and is not inactivated in the presence of homologous antiserum. In certain respects this inhibitor is comparable to "Interferon," a factor appearing in chick embryo tissues exposed to influenza virus.[8] That an inhibitor analogous to that described here may represent an essential determinant in chronic cell infection *in vitro* is suggested by the evidence presented. It is possible that an analogous factor may be produced *in vivo* in areas of infection. If so, it might play a role in the mechanisms of resistance, now largely unknown, that are operative during the acute stage of viral disease.

Summary.—Medium from human kidney tissue culture cells exposed to a strain of avirulent chick embryo-adapted Type II poliovirus inhibits the infection of human amnion and renal cells by homotypic and heterotypic poliovirus as well as other unrelated viruses. This property does not appear to be associated with infective virus or specific viral antigen.

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[1] A specimen of this virus designated here "RMC virus" according to the initials of the authors who first described it (Roca-Garcia, M., A. W. Moyer, and H. R. Cox, *Proc. Soc. Exp. Biol. & Med.*, **81**, 519 (1952) was kindly supplied by Dr. Herald R. Cox of the Lederle Laboratories.

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SOME STATISTICAL OBSERVATIONS ON A COOPERATIVE STUDY OF HUMAN PULMONARY PATHOLOGY. II

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In our first paper[1] we gave some general average data for the readings of eight pathologists in eight different cities on slides made from sections taken in standard positions in run-of-the-mill lungs at autopsy, using the following classifications: normal, hyperplasia, metaplasia, atypical metaplasia, carcinoma-in-situ and carcinoma. As carcinoma-in-situ was found so rarely by any of the pathologists, that classification will be combined with atypical metaplasia in this continuation of the study; there will be only five groups and their rank indices[2] will be 0, 1. 2, 3, 4.