Production of an Inhibitor Substance by DA Myxovirus. Auto-Inhibition of Plaque Formation.* (26937)

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It was reported(1) that a myxovirus, the DA virus, was detected by formation of plaques under an agar overlay but not by cytopathic effect (CPE) in parallel fluid cultures. Subsequently, it was noted that no DA virus plaques appeared in cultures inoculated with high concentrations of virus, but distinct plaques were observed in cultures inoculated with low virus concentrations. The present report is concerned with observations on this "prozone" type of reaction and the possible mechanisms involved in this phenomenon.

Materials and methods. Virus strain. DA virus, a newly isolated myxovirus, belongs to the parainfluenza group(2) but is not related to the 4 known prototypes. The isolation and characterization of this agent will be reported in detail elsewhere(3). *Cell cultures.* Rhesus monkey kidney cell monolayer cultures prepared in the usual manner, either in tubes or in 3 oz. prescription bottles, were used. All cultures were maintained in a medium containing 2% calf serum and 0.5% lactalbumin hydrolysate in Earle's salt solution. *Virus assay.* Virus infectivity titrations were determined in rhesus bottle cultures by the plaque technic(4). The hemadsorption technic described by Vogel and Shelokov(5) was also used in certain experiments. Both guinea pig and one-day old chick red blood cells were used in the hemagglutination test.

Results. The prozone reaction during serial passage of virus. Undiluted tissue culture fluids containing DA virus were used in making serial passages at weekly intervals over the last 2 years. Distinct CPE was not observed in the fluid cultures at any time. Infectivity titers of each passage level were determined by plaque titration. No plaques were evident in bottle cultures inoculated with undiluted or high concentrations of virus fluid, but clear distinct plaques were obtained in bottles inoculated with 10^{-4} and 10^{-5} dilutions of the same virus suspension. Although this "prozone" phenomenon was observed in each titration, there was no significant difference in infectivity titer end points between the 2nd and the 150th tissue culture passage levels. Fluids from later virus passages were also assayed by the hemagglutination test using chick erythrocytes, and no significant change in titer was noted, although minor variations were observed from time to time. Experiments to determine if the infectivity endpoints could be changed by serial passage of the virus in which low concentrations of DA virus were serially transferred, yielded the same results as those obtained using high concentrations of virus. On several occasions, the hemadsorption technic was also used for determining the infectivity titers as compared to the plaque method. The results were essentially the same by both methods.

Growth of DA virus in fluid cultures and under agar overlay. Growth rates of the DA virus in fluid cultures and under agar were compared, using undiluted virus suspensions as inocula (multiplicity of 0.1). After 2 hours' adsorption at 37°C, unadsorbed virus was removed and the infected cultures were washed, then divided into 2 groups. One group was overlaid with the agar-medium mixture, 10 ml per bottle, and fluid medium was added to the other group of cultures, also 10 ml per bottle. Daily samples from each group were removed from the incubator and the infected cultures with the media were frozen immediately at −70°C until assay. Prior to titration, the samples were thawed and the fluids centrifuged at 500 rpm for 10 minutes, to remove agar and cell debris. Infectivity titers of each sample were then determined by plaque titrations. Day *0* samples were taken on the day of inoculation, subsequent to addition of media. Virus multi-

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TABLE I. Growth of DA Virus in Fluid Medium and under Agar Overlay.

Days after inoculation*	Infectivity titers (log PFU/ml)	
	Fluid medium	Agar overlay
0	3.6	2.1
1	5.1	4.5
2	6.3	6.3
3	6.8	6.9
4	7.0	6.1
7	7.3	6.0
8	6.8	5.7
9	6.9	4.9

* Inoculum: 5.5 log PFU/bottle culture containing 3×10^6 cells.

plication occurred in both agar overlaid cultures and cultures maintained with fluid medium, although cellular changes (CPE or plaques) were not visible in either group (Table I). It was also noted that the virus titers obtained from the agar overlaid cultures were somewhat lower than titers obtained in fluid cultures, and it was possible that a part of the infective virus population under agar could have been lost during the process of removing the agar in order to recover the virus.

Development of hemagglutinin and occurrence of prozone reaction. Each of a series of rhesus tube cultures containing 3×10^5 cells was inoculated with 4×10^5 PFU of the DA virus. After 2 hours of adsorption at 37°C, the unadsorbed virus was removed, and 1 ml of maintenance medium was added to each tube. All infected cultures were returned to the incubator. At hourly, and then daily intervals 2 tubes were examined for CPE, and their fluids were harvested and pooled. Half of the fluid was frozen at −70°C until assayed for infectivity, and half was tested for hemagglutinin activity. The infected cell cultures were tested for virus hemadsorption by adding 0.2 ml of a 0.5% guinea pig red blood cell suspension. Virus infectivity was determined by plaque titrations. Growth curves and appearance of hemagglutinin activity are shown in Fig. 1. New infectious virus appeared at 3-4 hours after infection, and gradually increased without evidence of cytopathic changes in the cultures for as long as 3 days. Hemadsorption was first observed at 8 hours after infection, but hemagglutinin activity could not be detected in the fluids until 48 hours, increasing in titer thereafter. Fluids obtained from the first 24 hours induced no "prozone" effect. However, when harvests collected 48 to 72 hours after infection were plated, plaque formation was inhibited in the low dilutions. It was at this time, *i.e.*, 48 hours after infection, that hemagglutinin activity was detected.

Demonstration of interfering activity of DA virus infected culture fluids treated by various physical methods. Aliquots of undiluted tissue culture fluids of DA virus were treated by 1) heat at 56°C for 60 minutes; 2) ultraviolet irradiation for 30 seconds at 250 microwatts per sq. centimeter; 3) freezing and thawing 3 times; 4) filtration using a gradocol membrane of 130 mμ (APD), which was known to hold back the infectious virus; and 5) ultracentrifugation at 30,000 rpm for 3 hours. The treated samples and the untreated controls were tested for their interfering capacity against the DA virus. The results are shown in Table II. It is evident that no significant inhibition of plaque formation occurred when the treated virus fluids and the challenge virus were combined, held at room temperature for one hour, and then added to cultures. However, if the inoculum contained high concentrations of active virus particles as in the case of the sediment (Table II) which contained 4.6 log PFU of infectious virus, inhibition of plaque formation was noted. On the other hand, if the treated virus fluids were added to the cultures first,

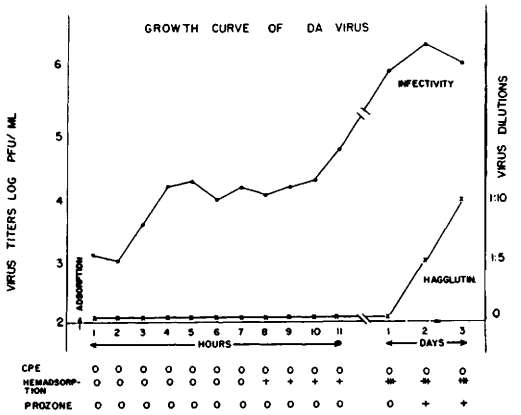

FIG. 1. Growth curve of DA virus in rhesus cell cultures, and development of hemagglutinin.

TABLE II. Interfering Activity of DA Virus Infected Tissue Culture Fluid.

Undiluted DA virus tissue culture fluid		Response to challenge with 50 PFU of infectious DA virus (avg No. plaques observed)	
Method of treatments	Infectivity titers (log PFU/ml)	Simultaneous inoculation*	One hr† adsorption before challenge
Untreated control	5.9	0	0
Treated by:			
(1) Heated 56°C	0	53	0
(2) UV irradiation	0	47‡	0
(3) Freezing and thawing	<.5	35	0
(4) Filtrate	0	18	0
(5) Ultracent. supernate	2.9	>100	0
" sediment	4.6	0	0

* Treated or untreated virus fluids and challenge virus were mixed and held at room temperature for 1 hr, then added to cultures simultaneously.

† Treated or untreated virus fluids were added to cultures and adsorbed for 1 hr at 37°C before challenge virus was added.

‡ Minute plaques.

and adsorbed one hour before DA virus challenge, plaque formation was prevented by all the various treated materials, regardless of the presence or absence of infectious virus in the interfering system (Table II).

Discussion. The "prozone" effect of plaque formation by DA virus in the present study may be attributed to a number of mechanisms. The presence of incomplete virus(6) seems unlikely, since the infectivity titers and hemagglutinin titers of the earlier and the later passages were not significantly different when undiluted virus fluids were used for the serial transfers. The presence of non-plaque forming particles such as those observed with the influenza viruses(7) was not possible since the infectivity titers obtained either by hemadsorption or by plaque technic were essentially the same. In addition DA virus originated from single plaques(1), and was purified through several plaque passages. The "prozone" reaction was observed even when the virus was obtained directly from a single plaque and plated.

The auto-suppression of plaque formation by DA virus would seem probably to be due, at least partially, to an inhibitor substance which, in most of its properties, resembled interferon(8) and related interfering substances(9-12). There was found to be a common mechanism of such inhibition, namely, 1) protection of cells from infection by stimulation of an inhibitor using high concentration of infectious virus, and 2) protection of cells from infection by introduction of the preformed virus-free inhibitor. In the latter case an adsorption of one hour or longer prior to challenge was necessary for maximum protection of the cells.

The finding of such an inhibitor substance does much to explain some of the unusual features of DA virus replication. When heavy inocula were used, no plaques were observed, yet it was found that with the same heavy inocula virus multiplication occurred to an almost equal extent, both under agar and in a fluid medium (Table I). It appears that complete inhibition of plaque formation was not due to a complete suppression of viral multiplication, but initiation of cellular infection was dependent upon the ratio between infectious virus and inhibitor substance. Therefore, plaque formation and CPE were not observed when the concentration of inhibitor in the inocula was high enough to suppress, at least partially, virus growth.

When high dilutions of DA virus were used, small amounts, if any, of inhibitor substance may have been transferred along with the virus, but such a limited amount of inhibitor apparently was not able to prevent the initiation of an infected area. As virus multiplication continued under agar, inhibitor substance was also produced, but the newly

produced inhibitor may well have diffused through the agar medium and thus had a negligible effect. Hence, in the immediate area of infection the ratio of infectious virus to virus inhibitor remained high and plaque formation could therefore continue. If, on the other hand, such an infected area was covered with fluid medium, then the inhibitor substance accumulated, and the ratio between infectious virus and inhibitor substance was lowered, probably reaching an equilibrium. In this instance, the cells were protected, at least partially, from destruction during viral multiplication, and therefore no CPE appeared.

Summary. Auto-inhibition of DA virus plaque formation was observed in tissue cultures during serial passages of this virus. An inhibitor (interferon-like) substance was found in the virus-free culture fluids. The presence of such an inhibitor substance seemed to be the determining factor for the 2 unusual phenomena observed with the DA virus: 1) plaque formation under agar overlay without CPE in parallel fluid cultures, and 2) "prozone" reaction in plaque formation.

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Effect of Chemotherapeutic Agents on Friend Virus-Induced Leukemia in Mice.* (26938)

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This study was undertaken to (a) establish methods for testing of chemotherapeutic agents against the Friend virus-induced leukemia (FVL) of mice(1) and (b) investigate the effect of a number of "established" and newer drugs.

Sugiura and Stock(2,3) have studied the effect of a variety of compounds on FVL using the spleen weight 3 weeks after virus inoculation as the criterion of evaluation. Effective compounds also were tested for their ability to increase survival time of infected animals. Mirand, Prentice, Hoffman and Grace(4) demonstrated that soon after inoculation, Friend virus produces a marked increase in uptake of Fe^{59} by the spleen. Moreover, this disease in HA/ICR Swiss mice is associated with malignant proliferation of the splenic reticulum, stimulation of the erythroid and myeloid elements of the spleen and liver, incerased hematocrit, presence of immature and mature erythrocytes in the blood, and leukocytosis(5,6).

Materials and methods. Adult mice (25-30 g), HA/ICR Swiss strain, were inoculated with the Friend virus† from a virus pool, originating from the ninth bi-weekly passage into Swiss mice in our laboratory, which was

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