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Received January 16, 1964. P.S.E.B.M., 1964, v116.

Potentiating Effect of Fractions of Eastern Equine Encephalomyelitis Virus on Interferon Production.*† (29193)

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Interferon is a soluble protein which has the interesting property of inhibiting intracellular virus replication. It is produced by cells exposed to any of a large group of viruses, both in infectious and inactivated forms(1,2). Earlier, it was found that heat inactivated Sindbis virus failed to mediate interferon production in chick cell cultures, but it increased such production when these cultures were subsequently infected with active virus(3). This potentiating effect of the non-infective virus we call "priming" of interferon production.

Attention is here directed to EEE virus, which is a pathogenic member of the same arbovirus group as Sindbis virus. In our hands, infective EEE virus is inconsistent in stimulating interferon production in chick cell cultures without priming(4). The present study was undertaken to elucidate the conditions under which priming takes place, and to define by density gradient centrifugation the components of EEE virus responsible for the priming activity.

Materials and methods. Stock suspensions of EEE virus (New Jersey) previously described(3) were purified by single plaque isolations 3 times and grown in chick embryo cell cultures (CECC). To prepare these cultures, cells obtained from 11-day-old embryos were suspended in 3 oz. bottles and nourished in 0.5% lactalbumin hydrolysate dissolved in Hanks' balanced salt solution (BSS) and 4% inactivated calf serum (LAH medium)(3). To prepare serum free virus suspensions, cells were overlaid with Eagle's basal amino acid and vitamin mixture in Hanks' BSS (E-H medium). Pellets of EEE virus were obtained by centrifuging harvests of stock virus titering about 10^8 p.f.u. per 0.1 ml at 78,000 $\times$ *g* for 3 hours at 4°C. The pellet material was suspended in E-H medium to a volume representing 2- to 100-fold of initial concentration.

For CsCl density gradient centrifugations: to 3.5 ml 25% (w/w) CsCl aqueous solution, 1.0 ml virus pellet suspension was added. The mixture was centrifuged at 100,000 $\times$ *g* for 24 hours. To collect fractions, the bottom of the tubes was pierced with a #24 needle, and successive drops were collected in a series of Wassermann tubes and diluted in E-H medium containing 5% calf serum.

For preparation of "primer" from serum-free virus suspensions or virus fractions in CsCl density gradients, 3.5 ml of such material in a 100 mm petri dish were first irradiated for one minute from a distance of 2 inches using ultraviolet light (UV) emitted from a 28.75 watt lamp with maximum emission at 2537 Å. Then the irradiated material was incubated at 37°C for 12 hours to completely eliminate viral infectivity. Excessive irradiation or heating was found to eliminate the priming effect.

Virus was titrated by plaque formation and interferon by plaque inhibition in bottle cultures. Interferon titers were expressed in percent reduction of plaque formation of 100-150 plaque forming units (p.f.u.) of EEE virus. From previous experience, an inhibition of less than 20% was not considered significant(3). In testing the interferon effect

* This study was supported by N.I.H. grant.

† Portions of this work were derived from Dr. Mahdy's doctoral dissertation.

TABLE I. Effect of "Primer" on Interferon Formation.

Hr of preincubation at 37°C	Primer plus infective virus* 1:1	1:4	1:8	Primer alone† 1:4	CF plus infective virus‡ 1:4
1	23§	37	18	<1	12
2	87	40	21	N.D.	1
3	92	46	38	N.D.	1
4	95	70	54	13	4
5	97	69	74	N.D.	7
8	98	77	84	N.D.	14

* Duplicate cultures each overlaid with 0.7 ml of "primer" in the form of inactivated EEE virus suspension were allowed to incubate for the period indicated; then decanted, washed with 5.0 ml Hanks' BSS, replenished with 5.0 ml LAH medium, and inoculated with 10^4 p.f.u. infective EEE virus. After 18 hours incubation, the fluids were collected, inactivated by UV irradiation for 6 minutes, and titrated by plaque inhibition for interferon activity in the 3 dilutions indicated.

† Treated as in (*), except no infective EEE virus was inoculated.

‡ Treated as in (*), except instead of "primer," a control fluid (CF) consisting of E-H medium was used.

§ Amount of interferon expressed in % reduction of 97 p.f.u. EEE virus inoculated. Inhibition <20% not considered significant. Each figure represents the mean of two bottles.

of fluids containing infective virus, infectivity was inactivated by heating at 56°C for 2 hours or by UV-irradiation for 6 minutes. Separate experiments indicated that EEE virus material inactivated in this manner contained negligible priming effect while maintaining interferon activity.

Results. A representative experiment demonstrating the effect of priming and the effect of length of incubation of cell cultures with "primer" on interferon and virus production is shown in Table I. It is apparent that no interferon is produced if primed cultures are not infected with active virus; confirming the impression that certain inactivated arboviruses alone do not produce interferon(3,5). In addition, infective virus alone does not produce significant interferon titers without priming. However, interferon is produced if primed cultures are infected. This priming effect increases with length of preincubation from 1 to 8 hours, with no significant increase in interferon formation unless cultures are primed for more than 2 hours.

To determine whether the priming activity of EEE virus is a property of the complete infectious particle or whether it resides in a distinct fraction, an EEE virus pellet in E-H medium representing an 80-fold concentration of the original harvest, and titering $10^{10.0}$ p.f.u. per 0.1 ml was centrifuged in CsCl (Fig. 1). The fractions collected were tested for viral infectivity and after inactivation of infectivity, for interferon priming effect as described under the caption of the Figure. It was found that 1) the peak infectivity was

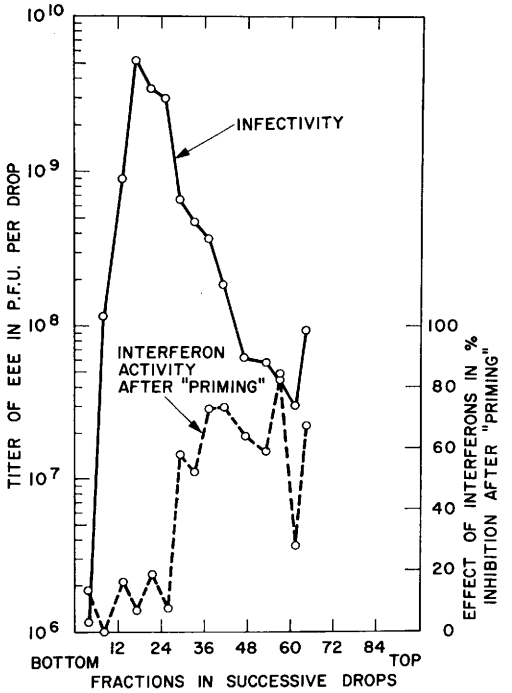

FIG. 1. Infectivity and interferon priming activity of fractions of EEE virus separated by CsCl density gradient centrifugation. Contents of entire tube totaled 62 drops. These were collected in 15 fractions each consisting of 4-6 drops (avg volume of one drop: .070 ml). Each fraction was diluted in 2.7 ml E-H medium with 5% calf serum. All fractions were tested for infectivity and for priming effect. To do the latter, 1.0 ml aliquots of a 1:10 dilution in E-H medium of the collected fractions were inactivated as described for primers and overlaid on single chick cultures for 18 hr at 37°C. After decanting and washing, 10^4 p.f.u. EEE virus were added to each culture along with 5 ml E-H medium, and harvests were collected in 24 hr, inactivated at 56°C for 2 hr and tested for interferon activity. Activities plotted represent averages of duplicate tests of 1:4 dilutions of such harvests. Control fluids from cultures receiving only infective virus but no primer showed <20% plaque inhibition.

found at the 4th fraction, corresponding to the 15th-17th drops. This corresponds to a density of 1.23, which is higher than the density of EEE virus reported by Taylor *et al* (6). 2) The priming activity is minimal in the peak infectivity fractions, but seems to be dispersed throughout the lower density fractions with peak activity in the 13th fraction, corresponding to the 53rd-56th drops, and a density of 1.12.

While results similar to the above have been repeatedly obtained, attempts to separate further the priming fractions by first partially purifying the virus pellet by Freon 112 and nuclease digestion before gradient centrifugation have been so far unsuccessful due to the instability of the priming fractions under these circumstances. Similarly, recycling of the effective fractions in various concentrations of CsCl has not resulted in more precise banding.

Discussion. It is apparent from extensive reports on systems of interferon production that both inactivated and infective virus play a role in producing interferon, depending on the virus and cell system used(2). So far, while inactivated arboviruses by themselves have not been shown to produce interferon (2,3,5), certain of these forms potentiate or prime its formation(3). The nature of this priming effect is unclear. Whether it is a necessary condition of interferon formation in such systems is also unclear. Thus it is well known that interferon formation may be mediated by infective arboviruses suspensions alone(2,3,4), the possibility exists that these suspensions may contain "primer" in the form of inactivated virus especially if they were inoculated in low dilutions. Primer may also be formed during incubation if high dilutions of infective suspensions are used(3).

The results of the present work show that with respect to EEE virus, the inactivated form also plays a definite role in interferon formation. This priming resides in low density virus fractions which are distinct from the fraction with peak infectivity. Possibly these low density fractions represent structurally different particles, but this remains to be supported by chemical and morphological evidence. Generally, better identification of the components of crude EEE virus suspension is indicated. An alternative interpretation is that the priming resides in the complete infectious particle, but high concentrations of this in the peak infective fractions may be directly toxic to cells or antagonistic to the priming effect. However, these fractions exerted no direct toxic effect on cells as far as could be ascertained morphologically, and dilutions of such fractions did not reveal masked priming activity.

This study also suggests the possible role of inactivated and/or subfractions of EEE virus in host defence. Presumably, a host or organ which produces EEE virus components that are good primers may be better protected against viral virulence.

Since further purification of EEE virus in our study did not result in better isolation of the priming factor, we cannot rule out cellular impurities playing a role in effective priming. However, it is unlikely that such impurities alone are responsible for priming because the priming effect is neutralized by antiserum obtained from a horse inoculated with EEE virus infected mouse brain.

The role of non-infective viral forms in interferon production has also been postulated in Newcastle disease virus(7), a member of a group of viruses well known for structural heterogeneity. These forms may be incomplete, or they may have structural peculiarities which underlie their ability to promote interferon formation in the cell. In view of the postulated role of nucleic acids in interferon formation(8), one simple possibility is that their RNA component may be more exposed and accessible.

Summary. Interferon production by infective EEE virus in chick cell cultures was potentiated by preincubation of cultures with inactivated EEE virus. Components responsible for this potentiating effect were obtained by CsCl equilibrium density gradient centrifugation of EEE virus and were found to be distinct from the fraction representing peak infectivity.

Thanks are due to Mrs. Sandra P. Mitchell for excellent technical assistance.

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Received January 23, 1964. P.S.E.B.M., 1964, v116.

Parathormone Bio-Assay of Plasma in Hypercalcemic Tumor Rabbits.* (29194)

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There is increase in plasma calcium and decrease in plasma phosphorus in rabbits with VX-2 carcinoma(1). Bone reabsorption has also been noted in tumor animals. Possibility that the tumor produces a parathormone-like substance seems likely. Lemon(2) has shown an increase of such substances in hypercalcemic patients with neoplastic disease. Assay of plasma from hypercalcemic tumor bearing rabbits for parathormone-like activity is reported here.

Procedure. Fifteen white New Zealand rabbits weighing 2.5 kg each were used. Two ml of VX-2 carcinoma tumor suspension were injected into right thigh of ten rabbits. Five rabbits served as controls. Heparinized blood samples were obtained weekly by ear vein drip method, immediately chilled with ice, and centrifuged in cold at 3000 rpm for fifteen minutes. Plasma was removed and stored at −10°C. Plasma calcium was determined by Skeggs modification(3) of Moser and Williams method(4).

Plasma was assayed for parathormone-like activity using a modification of method described by Lemon(2). White Swiss mice weighing 25 g were divided into 77 groups of 5 each. After a 4-hour fast each mouse was hydrated by intraperitoneal isotonic saline injection, 5% of body weight. Groups of mice were then treated as follows:

Three units parathormone, 8 groups.

One unit parathormone, 7 groups.

Plasma from hypercalcemic tumor rabbits, 22 groups.

Plasma from normal rabbits, 18 groups.

Five % dextrose solution, 19 groups.

Each mouse was injected with 0.25 ml of test substance subcutaneously. Fifteen minutes after injection each group was placed in a metabolic cage and urine collected separately from feces for 4 hours. Total 4-hour urinary phosphorus excretion was determined by method of Fiske and Subbarow(5).

Results. Total urinary phosphorus per group is shown in Fig. 1. There is an increase in phosphorus excreted by parathormone treated mice as compared to controls, with no significant difference between 3 unit and 1 unit dosage. Similarly, no significant difference exists in phosphorus excretion between control groups and mice receiving normal rabbit plasma. However, 8 of 22 groups treated with hypercalcemic (18.5 to 27.0 mg%) rabbit plasma excreted more urinary phosphorus than any groups receiving normal rabbit plasma. Analysis of difference of means in parathormone treated groups compared to control groups demonstrates statistically significant difference ($P<0.001$). An analysis of variance between groups treated with normal rabbit plasma and those receiving hypercalcemic rabbit plasma indicates a significant difference in variability between the two groups (null hypothesis $P \cong 0.02$).

Discussion. Parathormone bio-assay is based on increase in urinary excretion of phosphorus(6) after administration of exoge-

* Aided by NIH grant.