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Received May 18, 1965. P.S.E.B.M., 1965, v119.

Inhibition of Arboviruses by a Constituent of a Staphylococcus.* (30407)

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In the course of studies on the phagocytosis of inactivated staphylococci by primary monolayer cultures of human amnion cells (1) it was found that cells exposed to a suspension of these bacteria were resistant to Sindbis virus. Investigation of this phenomenon revealed that a constituent of the bacterium conferred protection by inhibiting viral multiplication. Experiments reported here attempted to explore the mode of action of this inhibitor and to define some of its physical and chemical properties.

Methods and materials. At the outset it was found that a constituent of several different strains of staphylococci inhibited multiplication of Sindbis virus. The bacterium employed in the experiments outlined below was recovered by Mrs. Dixie Kaslick (Dept. of Clinical Laboratories, Children's Hospital Medical Center) as a rare organism from the urine of a patient with cystic fibrosis. The organism was classified as a staphylococcus according to the criteria of Wilson and Miles (2). The various fermentation reactions and its growth anaerobically were compatible with this classification. It possessed characteristics of both a *Staphylococcus albus* and a *Staphylococcus aureus*. It was coagulase negative; it did not liquify gelatin; and it fermented mannitol only after 5 days. On blood agar plates or agar slants it produced a pale golden yellow pigment.

Preparation of the inhibitory factor. Bacteria were propagated in 2 liters of Difco beef heart infusion broth in a gyrotory shaker water bath at 37°C for 60-72 hours. At this time there were approximately 5×10^8 viable organisms per ml. Although slight viral inhibitory activity was demonstrated in the supernatant after centrifugation (3000 rpm for 30 minutes) of the culture fluid, only the inhibitory factor found to be associated with the bacteria was systematically investigated.‡ Accordingly, the bacterial sediment was suspended in phosphate buffer and centrifuged at 3000 rpm for 15 minutes. One-half ml of concentrated bacteria was resuspended in 4.5 ml of distilled water; 5 ml of #12 Ballotini glass beads were added, and the bacteria were disrupted at 4°C in a Mickle disintegrator.

* Supported in part by Grant AI-01992-08 from Nat. Inst. of Allergy & Infect. Dis., NIH, USPHS.

† Career Development Award, Nat. Inst. of Allergy & Infect. Dis., NIH, USPHS, Bethesda, Md.

‡ The total amount of inhibitor present in the 2 liters of supernatant exceeded that present in the 2.5 ml of sedimented bacteria. It was more practical, however, to obtain inhibitor of high titer from extraction of the smaller volume of bacteria than from concentration of the larger volume of supernatant.

The resulting mixture of bacteria and glass beads was passed through a sintered glass filter. The filtrate was centrifuged at 10,000 rpm for 15 minutes in a Spinco model L preparative centrifuge. The supernatant was filtered through a 0.45 μ millipore filter.

In some experiments bacteria were disrupted by sonic vibration or the inhibitor was obtained by adding acetone to concentrated bacteria, but the method of mechanical disruption yielded material of greater activity.

Assay of the inhibitor. The pale yellow filtrate was assayed for viral inhibitory activity in primary cultures of human amnion cells inoculated with 100-500 $TCID_{50}$ of Sindbis virus. Two-fold dilutions of the filtrate in 1 ml of regular medium(3) were added to each of 1-3 amnion cell cultures immediately before inoculation of virus. (In initial experiments the cultures were pretreated with inhibitor for 24 hours prior to viral inoculation.) Culture tubes were cotton stoppered and placed in a 5% CO_2-air incubator at 37°C to minimize changes in the pH of cell cultures. The inhibitory titer of a given preparation was expressed as the highest dilution§ in which less than an estimated 25% of the treated monolayer cell culture was affected by the challenge virus and more than 75% of the untreated control culture was destroyed.

Viruses. Sindbis virus: laboratory strain AR 339 (Dr. R. A. Taylor), has been serially propagated in primary monolayer cultures of chick embryo and human amnion cells (multiple passages). West Nile virus: strain Egypt 101 (Mrs. Joan Daniels), and Western equine encephalitis virus (Mrs. Joan Daniels) has also been passaged serially in primary cultures of chick embryo cells. Virus titers were determined by standard techniques employing tube cultures of either chick embryo cells(4) or human amnion cells(3). The infectivity titer of Sindbis virus in chick cells proved to be approximately 100 times higher than in the human cells.

Results. In the course of these experiments 14 lots of staphylococcal inhibitor were prepared. When assayed shortly after preparation, viral inhibitory titers (Sindbis virus) ranged between 1:40 and 1:2560; the median titer was 1:400. The titer fell by a factor of 2-4 when treated cultures were maintained for several days after the control cultures had been completely destroyed by the virus.

Continued presence of the inhibitor in the culture medium was necessary in order to confer protection on amnion cells. Thus preincubation of amnion cells with 32 units‖ of inhibitor for 24 hours did not inhibit Sindbis virus if the inhibitor were removed and the cell sheet were washed 2-3 times prior to viral challenge.

Preparations of inhibitor were not toxic for primary cultures of human amnion, human embryonic kidney, chick and mouse embryo cells, KB or BHK 21 cells at the usual dilutions (*e.g.*, $\geqq$1:40) employed in the assay system. At higher concentrations (*e.g.*, 1:10) slight cytoplasmic granularity was observed and the treated cultures produced less acid than untreated control cultures. However, as will be discussed later, other viruses multiplied in amnion cells treated with a 1:10 dilution of inhibitor and induced typical cytopathic effects. It seems unlikely, therefore, that inhibition of Sindbis virus in cells treated with this concentration of inhibitor depends upon a nonspecific "toxic" effect.

Mode of action of inhibitor. Neither viral hemagglutination nor infectivity was affected by preincubation of virus and inhibitor. Thus incubation of 256 units of inhibitor with 4 hemagglutinating units of Sindbis virus for one hour at 37°C did not affect agglutination of goose cells by the virus. Similarly the infectivity of 1 and 10 $TCID_{50}$ of Sindbis virus was not diminished by incubation with 64 units of inhibitor for one hour at 37°C.

There was no evidence that the inhibitor prevented penetration of human amnion cells by Sindbis virus. Monolayer cultures were exposed to 0.2 ml of a mixture of Sindbis virus (containing 1, 10 or 100 $TCID_{50}$ of virus) and either inhibitor (64 units) or control buffer solutions. After 2 hours at 37°C virus-inhibitor and virus-buffer mixtures were removed; the cell sheet was washed 4 times with 2 ml of Hanks' salt solution; nutrient

§ This dilution was considered as one unit of inhibitor.

‖ See *Methods and Materials.*

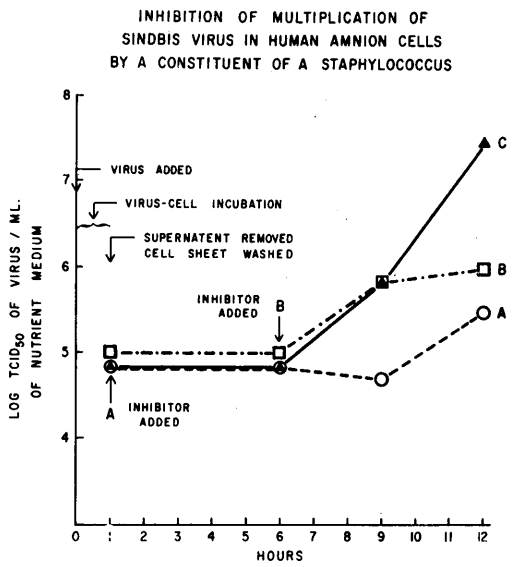

FIG. 1. Primary cultures of human amnion cells (200,000 cells/ml) were exposed to 0.2 ml of a dilution of Sindbis virus containing $10^{6.8}$ $TCID_{50}$ as assayed in chick embryo cell cultures. After incubation for 1 hr at 37°C the viral inoculum was removed and the cell sheet was washed 10 times with 2 ml of Hanks' salt solution and 2 ml of nutrient medium was added. An aliquot of the medium was harvested from all tubes for assay of residual virus. The tubes were cotton stoppered and placed in a 5% CO_2-air incubator at 37°C. Twenty units of inhibitor were added immediately after removal of the virus and washing of amnion cultures (A) and 6 hr later (B). There were 4 cultures in each group and 4 cultures in the control group (C). Aliquots of the medium from all cultures were harvested for viral assay at 6, 9 and 12 hr.

medium was added and the cultures were observed for CPE. No significant differences were noted between any of the cultures.

Dilutions of the inhibitor were usually added to amnion cultures immediately prior to viral inoculation. It was found, however, that addition of inhibitor to amnion cells at intervals *after* infection with Sindbis virus also suppressed viral multiplication. Preliminary experiments demonstrated that one cycle of Sindbis virus replication in human amnion cells required approximately 12 hours. In 3 experiments, inhibitor (12-32 units) was added 1 or 6 hours after exposing monolayer cultures to large quantities of virus. Aliquots of the nutrient medium were harvested for viral assay at intervals after infection from inhibitor-treated and control cultures. Significant suppression of viral multiplication occurred in all experiments (Fig. 1). In one experiment inhibitor was added 9 hours after viral inoculation and the increment of virus in the nutrient medium 3 hours later was log 0.3 compared to an increment of log 1.6 in control cultures during the same period.

The results of these experiments provide evidence that the staphylococcal inhibitor acts intracellularly. In order to determine whether the inhibitor blocked release of Sindbis virus from inhibitor-treated amnion cultures, the intracellular and extracellular viral content was determined. Thus in one experiment there were log 9.5 $TCID_{50}$ of virus per ml of medium from control amnion cultures after complete destruction by Sindbis virus had ensued. In comparable cultures protected by inhibitor, the extracellular viral titer was log 6.7 $TCID_{50}$/ml and the intracellular virus obtained by freezing and thawing the cells 3 times titered log 6.3 $TCID_{50}$/ml. The absence of a significant difference between intracellular and extracellular virus in 2 such experiments strongly suggested that the low virus titers in the nutrient medium of inhibitor-treated cultures was a reflection of depressed intracellular viral replication and not of interference with viral release.

Activity of inhibitor on Sindbis virus infection of primary cultures of human embryonic kidney, chick embryo and mouse embryo cells. The inhibitor also suppressed the CPE of Sindbis virus in primary cultures of human embryonic kidney cells. Although approximately 4 times less potent in these cells than in cultures of human amnion cells, a definite protective effect was readily demonstrated. However, preparations of inhibitor (containing 160-1280 units) were almost devoid of protective effect when assayed in primary cultures of chick and mouse embryo cells. In 4 experiments chick embryo cells were inoculated with only 10-32 $TCID_{50}$/ml of Sindbis virus and a transient inhibition of CPE was observed at inhibitor dilutions of 1:10-1:20. In 3 experiments 4-128 units of inhibitor did not protect mouse embryo cells inoculated with 10 and 100 $TCID_{50}$ of virus.

Effect of inhibitor on different viruses. It was found that the inhibitor conferred protection on human amnion cells inoculated

with 100 $TCID_{50}$ of either Western equine encephalitis (WEE) virus (a group A arbovirus) or West Nile (WN) virus (a group B arbovirus). In one experiment a given preparation of inhibitor was effective at a dilution of 1:640 against Sindbis, WN and WEE viruses, and in a second experiment a 1:1280 dilution of inhibitor suppressed the CPE of both Sindbis and West Nile viruses. In contrast to these findings, the staphylococcal inhibitor (16-32 units) did not diminish the cytopathogenicity of vaccinia, herpes simplex, Echo 2 and Echo 11 viruses, poliovirus I, adenovirus 7 and measles virus in cultures of human amnion cells.¶

Failure of inhibitor to induce the formation of interferon. It had been reported that statolon, an anionic polysaccharide, inhibited certain viruses by stimulating the production of interferon(5). It was of interest, since the staphylococcal inhibitor exhibits properties of a polysaccharide as shown below, to determine whether it acted in a similar manner, although the results of several experiments outlined above did not support this hypothesis. Ninety units of inhibitor or control buffer were incubated with human amnion cell cultures for 24 hours. The nutrient medium was then tested for interferon activity in other amnion cells, utilizing Echo 11 virus as the "challenge virus." No viral inhibition was observed at a 1:2 dilution of the medium. A "control" interferon prepared by infecting cultures of amnion cells with Sendai virus(6) inhibited the CPE of Echo 11 virus at a dilution of 1:32. Thus there was no evidence that the staphylococcal inhibitor stimulated the synthesis of interferon in human amnion cells.

In vivo *experiments with inhibitor.* The allantoic cavity of 7-day-old embryonated hens' eggs was inoculated with varying dilutions of Sindbis virus together with 40-80 units of inhibitor or control buffer. There was no evidence of any protection in 4 experiments. In 2 experiments the preparations of inhibitor even appeared to enhance the lethal effect of Sindbis virus.

Properties of the inhibitor. Various physico-chemical properties of the inhibitor are listed in Table I. Of interest was the resistance of the inhibitor to proteolytic enzymes, to nucleases and to ether. It was, however, affected by 0.1 molar sodium periodate. Thus in one experiment the inhibitor titer was reduced from 1:32 to less than 1:8 by treatment with sodium periodate.

Storage of the inhibitor for 1-2 months at 4°C often resulted in a 2-4-fold decrease in titer, and a far greater loss was noted after 6-8 months at this temperature. The inhibitor, however, was considerably more stable when maintained at —20°C or —70°C.

Purification of the inhibitor. Preliminary experiments suggested that the inhibitor might be concentrated and partially purified by addition of 95% ethanol. Nine volumes of ethanol were added to 1 volume of inhibitor and after 18 hours at 4°C a fluffy white precipitate formed. This was sedimented by

TABLE I. Physico-Chemical Properties of a Staphylococcal Inhibitor of Sindbis Virus.

Centrifugation 110,000 *g* 1 hr	0*
Dialysis 24 hr 4°C	0
Heat 60°C 1 hr	0
95°C 5 min	+
pH 2.2 18 hr 4°C	+
20% ether 18 hr 4°C	0
95% ethanol	0
Phenol	+
Sodium periodate 0.1 molar 1 hr 4°C†	+
Treatment with enzymes‡	
Trypsin, 500 γ/ml 1 hr 37°C	0
Chymotrypsin "	0
Papain "	0
RNA-ase "	0
DNA-ase "	0
Alpha amylase "	0
Neuraminidase, 1 hr 37°C	0

* 0 = inhibitory activity for Sindbis virus unaffected.
\+ = inhibitory activity for Sindbis virus reduced by more than 90%.

† The following were tested in this experiment: periodate + inhibitor; periodate + buffer; buffer + inhibitor. After incubation for 1 hr at 4°C, 0.5 ml of 40% glucose was added to each. The resulting mixtures were dialyzed 18 hr against 1 liter of distilled water at 4°C, and 5 hr against phosphate buffered saline prior to assay.

‡ Enzymes: Trypsin, chymotrypsin and papain —2 × crystalline, Nutritional Biochemicals (in employing papain 0.05 ml L-cysteine HCl (1 MM) was added). RNA-ase and DNA-ase — crystalline, Worthington Chemicals. Alpha amylase — Sigma Chemical (from *Bacillus subtilis* type II). Neuraminidase, Lot #3-1197 — titer 1:1000, Microbiological Associates.

¶ The viruses employed were all laboratory strains with long histories.

centrifugation at 3000 rpm for 30 minutes. The biologically inactive ethanol supernate was discarded, and the sediment was resuspended in phosphate buffer and dialyzed against distilled water and buffer. The resulting suspension was recentrifuged at 3000 rpm for 30 minutes and the clear supernatant proved inhibitory for Sindbis virus.

Discussion. Recent work undertaken in this laboratory by Carver and Naficy(7,8) and Naficy and Carver(9) has demonstrated that extracts of various bacteria and fungi inhibit certain animal viruses in cell culture. Each of these extracts was characterized by a narrow spectrum of antiviral activity. For example, a constituent of a corynebacterium (7,8) and *Penicillium cyclopium*(9) inhibited multiplication of representative arboviruses but did not affect other RNA or DNA viruses tested.

The viral inhibitor we have obtained from an undetermined species of staphylococcus by methods similar to those employed by Carver and Naficy(7,8) also revealed a narrow range of antiviral activity. It inhibited Sindbis, Western equine encephalitis and West Nile viruses but had no effect on vaccinia, herpes simplex, Echo 2 and Echo 11 viruses, poliovirus I, adenovirus 7 and measles virus. Like the arbovirus inhibitors of Carver and Naficy (7,8,9) it acted intracellularly. Thus addition of the inhibitor even 6 hours after exposing amnion cells to large quantities of virus significantly depressed viral replication in single cycle growth curve experiments.

In contrast to corynebacterium B1F(7) and to cyclopin(9) which inhibited Sindbis virus to comparable titers in both chick embryo and human amnion cells, the staphylococcal inhibitor was effective in primary cultures of human amnion and human embryonic kidney cells but afforded only transient protection of chick and mouse embryo cells infected with Sindbis virus. The relative lack of inhibition in these latter cells may depend upon their relative incapacity to take up the inhibitor or to destroy it intracellularly; but no experiments were undertaken to determine the factors responsible for this interesting difference.

In general the properties of the staphylococcal inhibitor as outlined in Table I were compatible with those of a polysaccharide. Biologic activity was not detectable after treatment with sodium periodate. The inhibitor was not affected by various proteolytic enzymes. In this connection it is of interest to point out that the inhibitors described by Carver and Naficy(7,8,9) were sensitive to the action of trypsin. It is probable, therefore, that the staphylococcal inhibitor belongs in general to those bacterial polysaccharides which have previously been shown to exhibit antivirus activity(10-13).

Intracellular antiviral specificity of various relatively nontoxic substances appears to be one of the most intriguing aspects of work in the field of viral inhibitors, not only from the potentially therapeutic usefulness of these substances but also as a means of more refined analysis of viral replication. Moreover, further work on the mode of action of these viral inhibitors may elucidate why some, such as the corynebacterium B1F(8) and the staphylococcal inhibitor we have described exhibit a marked protective effect against Sindbis virus *in vitro* but not *in vivo*, in contrast to others like cyclopin(9) which is not more potent *in vitro* than these inhibitors, yet is also active *in vivo*(14).

Summary. A constituent of an unidentified staphylococcus inhibited the multiplication and cytopathic effect of Sindbis, Western equine encephalitis and West Nile viruses in primary monolayer cultures of human amnion and human embryonic kidney cells. It did not inhibit other RNA and DNA viruses that were tested. Addition of the inhibitor 6 hours after infection of human amnion cell cultures with Sindbis virus significantly depressed viral replication. The inhibitor conferred only transient protection on primary monolayer cultures of chick and mouse embryo cells infected with Sindbis virus. The physico-chemical properties of the inhibitor that have so far been determined are compatible with those of a polysaccharide.

We gratefully acknowledge the constant help and advice of Dr. G. Michaels and Mrs. Dixie Kaslick of the Research Laboratory of the House of the Good Samaritan and the Department of Clinical Laboratories, Children's Hospital Medical Center. We are

indebted to Dr. John F. Enders for many pleasant and profitable hours of discussion and for his help in preparation of this manuscript.

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Received May 18, 1965. P.S.E.B.M., 1965, v119.

Plasma Factor Influencing Carbon Phagocytosis in the Isolated Perfused Rat Liver.* (30408)

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The important role of normal or immune serum opsonins in the preparation of bacteria for phagocytosis by leucocytes as well as by the fixed phagocytes of the reticuloendothelial system (RES) has been firmly established (1-4). However, the necessity of plasma factors for efficient phagocytosis of supposedly inert colloids is uncertain.

On the one hand, early experiments of Fenn(5) indicated that leucocyte phagocytosis of carbon and quartz particles was augmented by serum; more recently, Jenkin and Rowley(6) reported that humoral serum factors exert an important influence on intravascular colloidal carbon clearance. On the other hand, Biozzi *et al*(7) maintain that serum factors do not contribute in any way to the rate of phagocytosis of inert particles by the RES.

This paper presents evidence to substantiate the existence of a plasma factor capable of markedly affecting the rate of colloidal carbon gel phagocytosis by the Kupffer cells of the isolated, perfused rat liver.

Methods and procedure. Perfusion technique. The method of liver perfusion used in this study was essentially that described by Brauer(8) and Miller(9). Male rats of the Sprague-Dawley strain maintained on Purina pellets and water *ad lib* were used as both blood donors (rats of 400 to 600 g body weight) and liver donors (rats of 300 to 370 g body weight). The perfusate was either heparinized, whole rat blood or a semi-synthetic medium(10) described in Table I and supplemented with penicillin (20 U/ml) and streptomycin (0.025 mg/ml).

Livers with cannulae in the portal vein, thoracic vena cava and bile duct were rapidly extirpated, inserted into a constant environment chamber, and perfused under a hydrostatic pressure of 18 to 20 cm. Bile was collected and its flow was measured in a Wintrobe tube; blood flow was monitored by a flow tube inserted into the caval outflow.

Paired perfusions were performed by first perfusing one liver for 3 hours and then

* This investigation was supported by Research Grant HE 06588 from Nat. Heart Inst.

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