THE EFFECT OF *E. COLI* ENDOTOXIN ON THE INFECTIVITY OF WEST NILE VIRUS AND POLIOVIRUS

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VIRUSES AND ENDOTOXINS

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ABSTRACT

Administration of endotoxin prepared from E. coli O111:B4 (Difco) into mice prior to infection with West Nile virus (WN) enhanced the resistance of mice to infection with WN virus. The protective effect was manifested by a definite prolongation of the survival time. Prolongation of the survival time could be demonstrated in infections initiated by both large (10^5 LD_{50}) and small (100 LD_{50}) doses of virus inoculated I.C.

A single dose (37.5 mcg.) I.P. or I.C. of endotoxin inoculation 5, 9, or 24 hours prior to challenge with WN virus effectively protected mice against infection. However, variable protection was obtained by increasing the dose of endotoxin to 75 mcg. or decreasing to 3 mcg. The protective effect of endotoxin against infection was, in general, more apparent when a low concentration (100 LD_{50}) of challenge virus was used.

A prolongation of survival time was likewise demonstrated when the endotoxin was inoculated concurrently with challenge virus after keeping the admixture at room temperature for about 10 or 30 minutes. However, mice that had received WN virus 1, 2, or 3 hours prior to the administration of endotoxin, died earlier than the controls inoculated with

virus alone. Animal mortality was higher when endotoxin was administered I.P. instead of I.C.

Endotoxin, 150 mcg., had no suppressive effect on the replication of poliovirus type 1 in monkey kidney cell monolayers and did not alter the yield of the virus from these cells.

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INTRODUCTION

Endotoxins are defined as "large molecular weight substances that are present in the intact bacterial cell wall and which exhibit typical toxicity, pyrogenicity and antigenicity" (Ribi et al., 1962).

Bacterial endotoxins are heat stable substances but are labile to mild acid hydrolysis and are precipitable by alcohol, acetone or by the salting out procedures. One of the first attempts to extract endotoxins selectively from intact cell walls of gram-negative bacteria was the trichloroacetic acid method developed in 1933 by Boivin et al. This was followed by the phenol-water extraction method in 1952 by Westphal et al. Other modified processes were developed later (Ribi et al., 1964). The endotoxin obtained by these methods consisted mainly of polysaccharide with variable amounts of lipid, phosphorus and nitrogen. The chemical composition of the endotoxin depends greatly on the method of extraction, the strain of bacteria used and the medium in which these are grown (Fukushi et al., 1963; Ribi et al., 1964). Although the chemical composition of endotoxins obtained from different gram-negative bacteria may vary, yet the qualitative biochemical activity seems to remain constant. The term lipopolysaccharide has been widely accepted to represent endotoxin.

Effect of Bacterial Endotoxins on Virus Infections in Mice

In an attempt to determine the lethal dose of E. coli endotoxin in mice, Noyes et al. (1959) found that the amount of E. coli endotoxin required to kill mice was the same by intracerebral (I.C.) and intravenous (I.V.) routes of inoculations. However, the lethal dose was twice this quantity when injected intramuscularly (I.M.). After inoculation, the endotoxin appeared chiefly at the periphery of the liver lobule of the mouse and none was detected in the spleen. Noyes et al. (1959) found that bacterial endotoxins remained for a long time in the liver. They noted that one third of the original E. coli endotoxin injected was still found in the liver of mice at the end of one week. Attempts to detect endotoxin in the brain of animals after its inoculation I.V. even in massive doses have been unsuccessful (Noyes et al., 1959).

In mice, 24 hours after injection of endotoxin, there was an increase in the number of Kupfer cells in the liver and of macrophages in the spleen (Howard et al., 1958). An increased endotoxin clearing activity by the reticuloendothelial system (RES) also occurred (Boizzi et al., 1955). This RES stimulation was considered to be the reason for the appearance in the inoculated animals of a non-specific resistance to bacterial and viral infections (Meier and Kradolfer, 1956; Kradolfer et al., 1957). This effect was found to be

of short duration, it usually lasted for two weeks (Schaedler et al., 1962). Following endotoxin inoculation into mice there was an elevation in the preexisting antibody level (Michael et al., 1961), and the opsonic activity of the serum against bacteria and viruses was likewise increased (Rowley, 1960).

In newborn animals, however, an attempt to increase the pre-existing antibody titer by endotoxin inoculation failed (Miler et al., 1964). Nevertheless, a stimulation of the RES with subsequent increase in resistance to infection with bacteria and viruses was noticed. The RES was stimulated markedly only with doses of endotoxin that were higher than 500 mcg (Smith and Thomas, 1954; Witebsky, 1936).

Meier and Kradolfer (1956) and Kradolfer et al. (1957) studied the influence of bacterial endotoxin preparations upon the course of infection in mice infected with encephalomyocarditis (EMC) and ectromelia viruses. The survival times of mice infected with these viruses depended upon the dose of endotoxin and virus used, and upon the time interval between the inoculation of endotoxin and viruses. With the EMC virus, the survival time in mice was in general prolonged after one injection of endotoxin either 72 hours before virus inoculation or 24 hours after virus inoculation. Irregular results were obtained when ectromelia virus was used instead. In another study Dougherty and Groupé (1957) found that I.V. or

I.C. inoculation of E. coli endotoxin protected mice against challenge of lethal doses of influenza virus.

Wagner et al. (1959) found that the survival time of mice infected with EMC virus or with Eastern Equine Encephalitis (EEE) virus was somewhat prolonged when these animals were inoculated with single doses of purified endotoxin obtained from Salmonella abortus equi, administered I.P., I.V., or I.C., 24 hours before the infective virus dose. Likewise, an increase in survival time occurred when endotoxin was injected concomitantly with the challenge virus, or when a course of treatment of endotoxin was given 6 hours after I.P. inoculation of EEE virus.

The effect of bacterial endotoxin on ectromelia virus was studied by Gledhill (1959b). His results confirmed the work of Meier and Kradolfer (1956) and Kradolfer et al. (1957) which showed that endotoxins can either increase or decrease the severity of ectromelia infection of mice depending upon the dosage of virus and endotoxin injected. Decreasing the concentration of endotoxin injected into adult mice did not increase the severity of the virus infection but, on the contrary, induced an improved protection (Kradolfer et al., 1957).

It was observed that after injection into dogs of

endotoxin an endogenous pyrogens, distinct from endotoxin, appeared in the blood of these animals (Petersdorf and Bennett, 1957; Petersdorf *et al.*, 1957). This may explain the finding that inoculation into normal mice of serum from other mice bled 2 hours after treatment with endotoxin altered their resistance to subsequent infection with viruses. Such treatment was found in fact to have a protective effect against lethal doses of a mixture of mouse hepatitis virus and *Eperythrozoon coccoides* (Gledhill, 1959a).

Gledhill (1958) further demonstrated that mice infected with mouse hepatitis virus became more susceptible to the toxic effect of endotoxins from gram-negative bacteria. The LD_{50} of endotoxin for the infected mice was about 10-fold less than for the untreated animals. Maximal effect was exhibited one week after virus infection.

Youngner *et al.* (1964) demonstrated that upon injection of a large number of live, virulent organisms of *Brucella abortus* into chickens, a viral inhibitor appeared in the circulation. A similar substance was described in rabbits by HO (1964). Stinebring *et al.* (1964) described an interferon-like substance in mice following the inoculation of certain bacteria and *E. coli* endotoxin into these animals. This substance reached its maximum level after 2 hours. Later, this virus-inhibiting substance was described to be indis-

tinguishable from interferon and was found to possess similar properties (Hallum et al., 1965).

Effect of Bacterial Endotoxins on Viruses in Tissue Culture

Wagner et al. (1959) showed that pretreatment of chick embryo monolayers with purified endotoxin did not reduce the plaque count with EEE virus. Likar et al. (1959), however, found that the yield of type 1 poliovirus from monkey kidney monolayers was considerably reduced by such treatment. Treatment of such cells with endotoxin from Salmonella typhimurium or E. coli, prior to inoculation with poliovirus reduced the titer of the virus by 1000-fold. An attempt to confirm this work was unsuccessful (Gledhill, 1964). Murphy et al. (1961) found that the yield of type 1 poliovirus was somewhat reduced when virus was mixed with E. coli endotoxin prior to inoculation into cell cultures. However, the suppressive effect of the endotoxin was not sufficiently marked.

Experiments which utilize a large number of variables, including different doses of endotoxins, various types of viruses, cell cultures, species of animals and modes of inoculation, are likely to yield results which will be difficult to interpret. Thus, it is not suprising that opinions postulated by various investigators regarding virus-endotoxin relationship and infection in animals have been partly conflicting. It is the purpose of this thesis to confirm or

reject some of the findings of previous investigators regarding the effect of endotoxin on the course of experimental infection with viruses. In the present work only one type of endotoxin obtained from E. coli and two types of viruses, poliovirus and West Nile virus, were used for all determinations while varying the time and the sequence of the inoculations. Tests were carried out using both suckling mice and cell culture monolayers. The work reported in this thesis presents the results of these investigations.

MATERIALS AND METHODS

White Swiss Mice

White Swiss mice were obtained from United States Naval Research Unit No. 3, Cairo, Egypt, 1951, and bred in this laboratory.

Nutrient Media

Lactalbumin hydrolysate - BSS solution (0.5%)	85 ml.
Eagle's solution* - 10X	10 ml.
Glucose solution 10%	5 ml.
Calf serum	15 ml.
Penicillin	100 units/ml.
Streptomycin	100 mcg/ml.

Sodium bicarbonate solution (2.8%), added in sufficient quantity to produce a pH of 7.4.

The medium was filtered through Seitz filter, and incubated for 72 hours at 37°C to test sterility. Antibiotics were added just before use.

Maintenance Medium

Lactalbumin hydrolysate - BSS solution (0.5%)	90 ml.
Glucose solution 10%	5 ml.

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*Science (1955) 122: 501.

Calf serum ..... 5 ml.

Sodium bicarbonate solution (2.8%), added in sufficient quantity to produce a pH of 7.4.

Tissue Culture (TC) Medium Eagle (Difco)

TC medium Eagle was prepared according to the specifications of the manufacturer (Difco, Michigan, U.S.A.).

A liter of 10X solution in medium was prepared by dissolving 1 gm. TC vitamins, Eagle, dried (0879-24), and 2.2 gm. TC Amino Acids dried (0794), in 500 ml. TC Dextrose salt solution by vigorous agitation. TC glutamine 5%, 60 ml., (5789-60), and TC Bicarbonate solution 10%, 16.8 ml., were then added. The pH of the solution was adjusted to 6.7-6.9 with CO_2 when necessary. The combined solutions were mixed thoroughly, and sterilized through a Seitz filter.

Hanks' Balanced Salt Solution (BSS) (Merchant, Kahn, and Murphy, 1960)

Solution A.

Material	Amount	Preparation
NaCl	80.0 gm.	Dissolve in 800 ml. dis-
KCl	4.0 gm.	tilled water.
$MgSO_4 \cdot 7H_2O$	2.0 gm.	
$Na_2HPO_4 \cdot 2H_2O$	0.48 gm.	
Glucose	10.00 gm.	

Material	Amount	Preparation
KH_2PO_4	0.6 gm.	
Solution B.		
$CaCl_2$	1.4 gm.	Dissolve in 100 ml. distilled water.
Solution C.		
Phenol Red	0.4 gm.	Mix phenol red in small amount of water until a paste, dilute to 150 ml. with distilled water, titrated to pH 7 with N/20 NaOH. Make up to final volume of 200 ml. Preserve with 1-2 ml. chloroform.

A. 10X Hanks' Stock Solution

Solution C, 100 ml. was added to Solution A and the volume was brought up to 1000 ml. by addition of Solution B. The mixture was then poured into a glass stoppered bottle and 3-4 ml. chloroform was added as preservative.

B. Working Solution

The working BSS was prepared by diluting 10X

stock solution with distilled water, dispensed in convenient size screw-cap bottles, and autoclaved at 120°C for 15 minutes. Next, sterile bicarbonate solution, 2-5 ml. was added aseptically to each 100 ml. of BSS.

Lactalbumin Hydrolysate - BSS (Merchant, Kahn, and Murphy, 1960)

In 100 ml. of working solution of BSS, 0.5 gm. of lactalbumin hydrolysate powder was dissolved. It was then filtered, autoclaved at 120°C for 15 minutes and stored at 4°C.

GKN Solution (Merchant, Kahn, and Murphy, 1960)

A. 10X GKN Stock Solution

Glucose	20 gm.
Sodium Chloride	160 gm.
Potassium Chloride	4 gm.

Dissolve all in 2000 ml. of distilled water.

Add 40 ml. of 1% phenol red and 6-8 ml. of chloroform and store at 4°C.

B. Working Solution

The stock solution was diluted 10-fold with bi-distilled water, autoclaved at 15 lb. pressure for 15 minutes and stored at 4°C.

Glucose Solution 10%

Dextrose 10 gm.

Bi-distilled water added in sufficient quantity to

make 100 ml., autoclaved at 15 lb. pressure for 15 minutes and stored at 4°C.

Trypsin Solution

Trypsin (1:250) vials* were used to prepare the trypsin solution. Solutions of 0.25% trypsin in GKN were prepared directly from the vials. This was used for dispersing the monkey kidney cells (Dulbecco, 1952). Before use the pH was adjusted at 7.8.

Tissue Culture Bottles and Tubes

The TC tubes and bottles used for these determinations were obtained from Kimble Glass Company U.S.A.

Calf Serum

Calf blood was obtained from a slaughter house in the vicinity of Beirut. The blood was kept at room temperature for few hours, the clot was then broken and the bottles were kept overnight at 4°C. Next day, the serum was separated from the clot by centrifugation, filtered through Seitz filter, inactivated for 35 minutes at 60°C, and kept at -20°C until used.

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*Trypsin 1:250, Microbiological Associates Inc. Bethesda, Maryland, U.S.A.

Bovine Albumin, Fraction V, Sterile 30% (Casals and Reeves, 1959)

Bovine albumin was obtained from the Nutritional Biochemicals Corporation (Cleveland, Ohio).

Endotoxin Preparation (Murphy and Wisner, 1962)

E. coli lipopolysaccharide 0111:B4 (Difco)*, 100 mgm., was dissolved in 10 ml. of sterile Hanks' balanced salt solution (BSS), sterilized by filtration through sintered glass filter, and stored at -20°C until used.

West Nile Virus (AR-248, M-875)

Stock virus was prepared by I.C. inoculation into 2-3-day old mice of 0.03 ml. of a 10^{-1} virus suspension (Smithburn et al., 1940). On paralysis or death, which occurred 3-4 days after inoculation, a 20% suspension of the brain was made, using 2% bovine albumin in phosphate buffer at pH 8 as diluent. Suspensions were tested for sterility and stored at -70°C until used. The virus suspension was titrated in suckling mice by I.C. inoculation using 10-fold dilutions, 10^{-1} - 10^{-9}, prepared in Hanks' balanced salt solution, pH 9.

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*Difco Laboratories, Detroit, Michigan, U.S.A.

Poliovirus Type 1

Sabin strain of poliovirus, type 1, was kindly supplied by Dr. T. Frothingham, Tulane University, School of Medicine, New Orleans, U.S.A.

Monkey Kidney Cells

These cells were kindly supplied by Dr. Chu of El-Fanar Laboratories, Lebanon. Originally it was obtained from the Near East Animal Health Institute, Iran Unit.

All mice used in these experiments were 2-day old Swiss mice, and each litter of mice consisted of at least 8 mice. All inoculations were made by means of 27 gauge needles using 0.03 ml. quantities per mouse for both I.C. and I.P. inoculations. Mice were observed closely after inoculation. Deaths in mice occurring within 48 hours after inoculation were considered to be due to bacterial contamination or to damage caused by the needle. No antibiotics were used in any of the experiments and sterility tests were carried out regularly on both virus suspensions and endotoxin solutions.

RESULTS

I. The Effect of Endotoxin on West Nile Virus Infection in Suckling Mice

The inoculation schedule of mice treated with endotoxin and West Nile Virus is presented in Table 1.

The mice that received 75 mcg. of endotoxin followed 24 hours later by inoculation of 100 LD_{50} virus showed some resistance to the lethal effect of WN virus. The mortality of endotoxin-treated mice was only 40% at the time when mortality of virus-inoculated control mice was 100% (Table 2). Furthermore, endotoxin-treated mice survived 16 hours longer than mice inoculated with virus alone (Table 3).

Mice that received 37.5 mcg. of endotoxin I.C. at intervals of 5, 9 or 24 hours prior to 100 LD_{50} virus administration, died approximately 23 hours later than the virus-inoculated control mice (Table 3). However, the percentage mortality was least when the interval between endotoxin inoculation and WN virus inoculation was 24 hours (Table 2).

Mice that received 3 mcg. of endotoxin 24 hours prior to inoculation of 100 LD_{50} virus survived 21 hours

longer than the mice inoculated with virus alone (Table 3). The mortality of endotoxin-treated mice was only 62% at the time when mortality of virus-inoculated control mice was 100% (Table 2).

Mice that received the 37.5 mcg. endotoxin I.P. at different intervals of 5, 9 or 24 hours prior to inoculation of 100 LD_{50} virus, showed a prolongation of survival time as compared with control mice inoculated with virus alone. The most pronounced effect of the endotoxin induced resistance was obtained when mice were inoculated with endotoxin 9 hours before WN virus challenge (Tables 2 and 3).

The most effective concentration of endotoxin in protecting mice against virus infection was 37.5 mcg. Reducing the dose to 3 mcg. induced equal protection. However, increasing the dose to 75 mcg. significantly reduced the protective effect as compared with 37.5 mcg. of endotoxin. The protective effect was the same when endotoxin (37.5 mcg.) was administered I.C. 5, 9 or 24 hours prior to challenge virus. However, endotoxin inoculated I.P. 9 hours prior to challenge virus gave better protection than when inoculated 5 or 24 hours prior to such challenge. Endotoxin administered I.C. 5 or 24 hours prior to virus inoculation was more protective than endotoxin administered at the same time interval via the I.P. route. However, the reverse was true when endotoxin was inoculated 9 hours prior to virus challenge.

The endotoxin, 37.5 mcg., administered 24 hours before inoculation of 10^5 LD_{50} West Nile virus induced some resistance to mice against infection with virus. Thus, while the mortality rate in virus-inoculated mice was 100% the mortality rate in the endotoxin-treated mice was only 50% (Table 2). The endotoxin-treated mice survived 16 hours longer than mice inoculated with virus alone (Table 3). Endotoxin had better protective effect against low doses (10^2 LD_{50}) of challenge virus than against higher doses (10^5 LD_{50}).

Mice that had received the endotoxin, 3 mcg., 24 hours prior to inoculation of 10^5 LD_{50} virus, survived 18 hours longer than mice inoculated with virus alone (Table 3).

There was a prolongation in survival time in mice that received an admixture of 75 mcg. endotoxin and 100 LD_{50} WN virus as compared with survival time of mice that received the virus alone. The effect was more pronounced when the endotoxin was kept for 30 minutes at room temperature in contact with the virus (Tables 4 and 5). The prolongation in survival time of mice inoculated with 100 LD_{50} virus and 37.5 mcg. endotoxin admixtures was longer than in mice that received the virus alone (Tables 4 and 5). Likewise, there was an increase in survival time in mice that received an admixture 100 LD_{50} virus and 19 mcg. of endotoxin as compared with mice that received the virus alone (Tables 4 and 5). There was a complete protection in mice when the endotoxin was kept for 30 minutes in contact

with the challenge virus. It was apparent that admixtures containing 75 mcg. or 19 mcg. of endotoxin and virus kept for 30 minutes at room-temperature were more effective in protecting mice than similar admixtures kept for 10 minutes only. Admixtures containing 19 mcg. or 37.5 mcg. of endotoxin and virus kept for 10 minutes at room-temperature were found to be superior in protecting mice as compared with admixtures containing 75 mcg. Moreover, the admixture containing 19 mcg. endotoxin and virus kept for 30 minutes at room temperature were found to have superior protective effect than the admixtures containing 37.5 mcg. or 75 mcg. and virus kept under the same conditions.

Mice that received the virus 1 hour, 2 hours and 3 hours prior to I.C. administration of endotoxin, died earlier than the virus controls. However, the percentage mortality of mice was lowest when the endotoxin was inoculated 3 hours after the virus challenge (Table 6). Likewise, mice that received the virus 1 hour, 2 hours and 3 hours before I.P. administration of endotoxin, died earlier than the virus controls. The percentage mortality in the mice that received the inoculation of endotoxin I.P. was again lowest when the endotoxin was inoculated 3 hours after the virus challenge (Table 6). This value was higher when the inoculation was done I.C.

TABLE 1

Inoculation schedule of mice treated with endotoxin and West Nile virus

	Endotoxin			West Nile virus*	
	Dose	Time of administration	Route of** inoculation	Dose	Time of inoculation
A.	75 mcg/0.03 ml.	Before virus inoculation	I.C.	10^2 LD_{50}	24 hrs after endotoxin inoculation
	37.5 mcg/0.03 ml.	Before virus inoculation	I.C.	10^2 LD_{50}	5, 9 and 24 hrs after endotoxin inoculation
	3 mcg/0.03 ml.	Before virus inoculation	I.C.	10^2 LD_{50}	24 hrs after endotoxin inoculation
	37.5 mcg/0.03 ml.	Before virus inoculation	I.P.	10^2 LD_{50}	5, 9 and 24 hrs after endotoxin inoculation
	37.5 mcg/0.03 ml.	Before virus inoculation	I.C.	10^5 LD_{50}	24 hrs after endotoxin inoculation
	3 mcg/0.03 ml.	Before virus inoculation	I.C.	10^5 LD_{50}	24 hrs after endotoxin inoculation
B.	75 mcg/0.03 ml.	Together with virus as admixture***	I.C.	10^2 LD_{50}	Together with virus as admixture
	37.5 mcg/0.03 ml.	Together with virus as admixture***	I.C.	10^2 LD_{50}	Together with virus as admixture
	19 mcg/0.03 ml.	Together with virus as admixture***	I.C.	10^2 LD_{50}	Together with virus as admixture
C.	37.5 mcg/0.03 ml.	1, 2 and 3 hours after virus inoculation	I.C.	10^2 LD_{50}	Before endotoxin inoculation
	37.5 mcg/0.03 ml.	1, 2 and 3 hours after virus inoculation	I.P.	10^2 LD_{50}	Before endotoxin inoculation

*All virus challenge was made I.C. and controls received same amount of virus.

**Two litters of mice were used for each experiment.

***Kept at room temperature for 10 and 30 minutes before injecting into mice.

TABLE 2

Mortality rate in virus inoculated mice pre-treated with endotoxin

Time of virus inoculation after endotoxin treatment	Per cent mortality* in virus inoculated mice pre-treated with endotoxin in concentrations of:				WN virus dose
	75 mcg. I.C.	37.5 mcg. I.C.	3 mcg. I.C.	37.5 mcg. I.P.	
5 hours	ND	40	ND	16.7	10^2 LD_{50}
9 hours	ND	33	ND	0	10^2 LD_{50}
24 hours	40	20	62	20	10^2 LD_{50}
24 hours	ND	50	50	ND	10^5 LD_{50}

ND = Not done

*Mortality rate in mice inoculated with virus alone = 100 per cent.

TABLE 3

Survival time in virus inoculated mice pre-treated with endotoxin

Time of virus inoculation after endotoxin treatment	Survival time* in virus inoculated mice pre-treated with endotoxin in concentrations of:				WN virus dose
	75 mcg. I.C.	37.5 mcg. I.C.	3 mcg. I.C.	37.5 mcg. I.P.	
5 hours	ND	23 hours	ND	16	10^2 LD_{50}
9 hours	ND	23 hours	ND	45	10^2 LD_{50}
24 hours	16 hours	23 hours	21 hours	12	10^2 LD_{50}
24 hours	ND	16 hours	18 hours	ND	10^5 LD_{50}

ND = Not done

*After death of all control mice inoculated with virus alone.

TABLE 4

Mortality rate in mice inoculated with admixtures of endotoxin and WN virus

Time of storrage of admixture at room temperature	Percentage mortality* in mice inoculated with admixtures of 100 LD_{50} WN virus and endotoxin in concentrations of:		
	75 mcg. endotoxin	37.5 mcg. endotoxin	19 mcg. endotoxin
10 minutes	40	37	20
30 minutes	25	0	0

*Mortality rate in mice inoculated with virus alone = 100 per cent.

TABLE 5

Survival time in mice inoculated with admixtures of endotoxin and WN virus

Time of storrage of admixture at room temperature	Survival time* in mice inoculated with admixtures of 100 LD_{50} WN virus and endotoxin in concentrations of:		
	75 mcg.	37.5 mcg.	19 mcg.
10 minutes	12 hours	28 hours	24 hours
30 minutes	31 hours	32 hours	57 hours

*After death of all control mice inoculated with virus alone

TABLE 6

Mortality rate in mice inoculated with WN virus prior to treatment with endotoxin

Time of endotoxin inoculation after virus treatment	WN virus I.C.	Endotoxin 37.5 mcg. I.C.	Endotoxin 37.5 mcg. I.P.
	Per cent mortality*	Per cent mortality*	Per cent mortality*
1 hour	0	87.5	100
2 hours	0	85	71
3 hours	0	12.5	50

*99 hours after virus inoculation.

II. The Effect of Endotoxin on the Response of Cells in Monolayer Culture to Infection with Poliovirus Type 1.

Endotoxin followed by poliovirus type 1.

Monkey kidney cell cultures in monolayers were prepared in tubes. Endotoxin, 150 mcg. was then added to the maintenance medium (MM) of 3-day old monolayer tubes. For the next 2 days the medium was removed daily and replaced by fresh MM containing same concentration of endotoxin. Three days after the addition of endotoxin they were infected with poliovirus type 1, 100 TCD_{50}. Fresh endotoxin was not added to cell cultures after they were infected with poliovirus. Monkey kidney monolayer tubes containing cell cultures infected with 100 TCD_{50} poliovirus type 1 and not treated with endotoxin, were kept as control.

Endotoxin and poliovirus type 1 admixture.

Monkey kidney cell cultures in monolayers were prepared in tubes. Endotoxin, 150 mcg., was then mixed with poliovirus type 1 100 TCD_{50}, and stored for 30 minutes at room temperature before being added to the 3-day old MK monolayer tubes. Other monkey kidney monolayer tubes were treated with poliovirus type 1 alone and kept as control.

Poliovirus type 1 followed by endotoxin.

Three-day old monolayer tubes of monkey kidney cell cultures were infected with poliovirus type 1. Thirty minutes later half the tubes were treated with MM containing 150 mcg. of fresh endotoxin and the rest were kept as control.

The fluid of 4-6 endotoxin-treated cell cultures and of control cultures were harvested at intervals of 12 hours (Likar et al., 1959), pooled and titrated.

In these experiments no antibiotics were added to the maintenance medium. Sterility tests were carried out on all endotoxin solutions, growth media and MM. Serum used for MM was tested and found to be free from poliovirus type 1 antibodies by neutralization tests.

Virus titers of supernates from endotoxin-treated cells were found to be identical to those of virus controls. This indicates that, under present experimental conditions, the treatment of monolayer cell cultures with endotoxin before, together or after infection with poliovirus type 1 had no suppressive effect on the replication of virus and did not alter the yield of the virus.

DISCUSSION

The results of the experiments reported herein indicate that when endotoxin was administered before the challenge virus it induced a protective effect in mice against subsequent inoculation with virus. The protective effect was manifested by a definite prolongation of the survival time in endotoxin-pretreated mice. Similar effects were observed when virus-endotoxin admixtures were inoculated into these animals. Contrariwise, when endotoxin was administered after the virus, the endotoxin-treated mice died earlier than virus controls.

It was further established that treatment of monolayer cell cultures with endotoxin before, together or after infection with poliovirus type 1 had no suppressive effect on the replication of virus and did not alter the yield of the virus.

Some of these results are in agreement with the findings of several other investigators. For example, Meier and Kradolfer (1956) and Kradolfer et al. (1957) observed that non-specific resistance was induced when endotoxin was inoculated into mice 72 hours before the administration of encephalomyocarditis (30 ID_{50}) or ectromelia (30 ID_{50}) viruses. In a similar study Dougherty and Groupé (1957)

showed that a single I.C. or I.P. inoculation of E. coli endotoxin, given 6 or 24 hours before influenza virus, induced a protection against the virus. In other studies, Wagner et al. (1957), demonstrated that the survival time of mice infected with encephalomyocarditis or EEE virus was generally prolonged when a single dose of purified endotoxin from Sal. abortus equi was inoculated I.C., I.P. or I.V., 24 hours prior to challenge virus. Likewise, Gledhill (1959b) observed that mice inoculated with endotoxin 24 hours prior to I.P. injection of ectromelia virus survived longer than the virus controls.

In later experiments Wagner et al. (1959) further indicated that a prolongation of survival time in mice occurred when the animals were inoculated I.P. with an admixture of 5 mcg. of endotoxin and 12 LD_{50} encephalomyocarditis virus. Observations made by Kradolfer et al. (1957), indicate that the effect of endotoxin upon virus infectivity may be dependent upon the dose of endotoxin and virus utilized in the tests and upon the time interval between the inoculation of the endotoxin and the virus.

The mechanism behind an increased resistance of mice to viral infection following administration of endotoxin has been a subject of much controversy. Wagner et al. (1957) suggested that the reason behind this increased resistance

was a moderate inhibition of the virus replication by the endotoxin. Gledhill (1959b), however, considered it to be due to a stimulation of the reticulo-endothelial system (RES) brought about by the endotoxin. He claimed that the stimulated RES clears the blood of virus more efficiently than the normal RES, and thereby lessens the invasion of vital structures such as the parenchyma of liver.

In 1964, Gledhill, in another attempt to explain the mechanism of non-specific resistance, described an endogenous sparing substance in the serum of mice following administration of endotoxin. This substance was found to inhibit certain viruses when inoculated together with the virus. Gledhill suggested that the presence of this sparing substance in the blood could be an additional factor in enhancing the resistance of animals to viral infections. Indeed, the fact that mice inoculated with virus-endotoxin admixtures were partly protected against the lethal effect of the virus, lends support to the hypothesis offered by Gledhill (1964). Apparently the endogenous sparing substance does not inactivate the virus directly. It has been considered to raise the resistance of mice in some undefined way either by modifying the virus-infected cells to produce fewer infectious particles, or by making normally susceptible cells more resistant to infection.

In these experiments it was quite apparent that mice inoculated with virus-endotoxin admixtures kept for 30 minutes at room temperature before inoculation, survived for a longer period of time than those inoculated with admixtures that were kept for 10 minutes only. This may indicate the possibility that such treatment results in a moderate inhibition of viral replication either through direct action of endotoxin on the virus or through enhancement of phagocytic defense. An explanation similar to this has been suggested by Wagner et al. (1957).

It may also be assumed that administration of endotoxin alone or in admixtures with virus stimulates the inoculated animal to release interferon in the circulation. Youngner et al. (1964) demonstrated that upon injection of a large number of live, virulent organisms of Brucella abortus in chickens, a viral-inhibitor appeared in the circulation. A similar substance was described in rabbits by HO (1964). Likewise, Stinebring et al. (1964) described a substance which appeared in mice following the inoculation of certain bacteria and E. coli endotoxin in these animals. It reached its maximum level after 2 hours. Later, this virus-inhibiting substance was described to be indistinguishable from interferon and was found to possess similar virus-inhibiting properties (Hallum et al., 1965).

In these experiments it was observed that when WN virus was inoculated into mice 1, 2 or 3 hours before the endotoxin no protection occurred. This suggested that the virus-inhibiting substance, which possibly appeared after adsorption, penetration and replication of the virus into cells, failed to protect the mice against infection. This may lead to the assumption that the virus-inhibiting substance produced as a result of endotoxin inoculation is analogous in its action to interferon as suggested by Hallum et al. (1965).

Sub-lethal doses of endotoxin administered I.C. or I.P. into mice after 100 LD_{50} of WN virus inoculation, did not induce any protection against infection with WN virus. Contrariwise, mice that received the endotoxin after the virus inoculation died before those that had received the virus alone. The reason for such behavior is not clear. It has been observed by Gledhill (1958) that mice pre-infected with mouse hepatitis virus became more susceptible to the toxic effect of lipopolysaccharide. The LD_{50} of lipopolysaccharide for virus-treated mice was about 10- to 100-fold less than for normal mice. Barlow (1964) demonstrated that during the incubation period following I.C. inoculation with lymphocytic choriomeningitis virus, mice were much more susceptible to I.P. inoculation of endotoxin than the uninfected animals.

Supernatent fluids from untreated cell cultures inoculated with poliovirus type 1, and endotoxin-treated cell cultures likewise inoculated showed identical virus yield upon titration. These results were similar to those obtained by Cohen and Gledhill (Gledhill, 1964) but differed from those of Likar *et al.* (1959). Likar *et al.* claimed that the yield of poliovirus type 1 could be suppressed by treatment of tissue cultures with endotoxins. Other findings have shown that pre-treatment of chick embryo cells with purified endotoxin from *Sal. abortus* did not effect the plaque counts of EEE virus (Wagner *et al.*, 1959). Nevertheless, Murphy and Wisner, (1962), pointed out that the effect of *E. coli* endotoxin on yield of poliovirus type 1 was slightly suppressive when virus and endotoxin were added concurrently into cell cultures.

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