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Effect of Westphal Lipid A on Viral Activities in Mice and Hamsters. (27563)

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Endotoxins of diverse microbial origin have long been recognized to exhibit a striking array of pathophysiologic effects(1-6) in animals, including enhanced resistance to infection with a variety of microbial agents. Chemically, the endotoxins are protein-lipid-polysaccharide complexes of high molecular weight. It is generally agreed that biologic activity of the endotoxin resides in the lipopolysaccharide part of the endotoxic complex. There is currently disagreement, however, as to whether biologic activity is resident in the lipid or in the polysaccharide component. Westphal(5), by acid hydrolysis, obtained a fraction from endotoxin called "Lipid A" which he believes is the active principle of the molecule even though it represents but a small fraction of initial total activity. By contrast, Ribi *et al.*(7) have obtained a fraction of endotoxin of undiminished activity which is mainly carbohydrate and which contains only a small residuum of lipid.

Endotoxins present in whole bacteria, in bacterial filtrates, or in bacterial extracts have been shown (8-15) to possess antiviral activities which include suppression of neurotoxicity of concentrated influenza virus (not due to viral proliferation), and of inhibition of pathogenicity or proliferation of ectromelia, encephalomyocarditis group or eastern equine encephalomyocarditis viruses. There have been no reports, however, of the activity of lipid A against viruses. Findings in the present study show that lipid A derived from *E. coli*, like whole endotoxin, induces resistance in animal hosts against viral toxicity and affords a slight protection against clinical consequences of certain viral infections.

Materials and methods. Lipid A and endotoxin of Escherichia coli were prepared* according to the general procedures of Westphal(5,16,17). The materials were dispensed in an aqueous solution of 1% Emulphor.† Endotoxin and lipid A fractions used in the studies were active in promoting host resistance in mice against *Klebsiella pneumoniae*. The Emulphor was inert in all tests. The *cholera filtrate* was prepared according to procedures described earlier(18) and was found to be active in destroying receptors of red blood cells and non-specific inhibitors in mammalian sera. *Viruses.* All viruses employed were from the inventory of documented strains maintained in this laboratory. Influenza A virus strain NWS (neurotropic) was propagated in embryonated hen's eggs. Pneumonia virus of mice (PVM) was propagated in lungs of weanling mice. Encephalomyocarditis strain Columbia SK, herpes simplex and poliovirus II (Lansing, mouse adapted), viruses were propagated in mice inoculated intracerebrally. The hamster adapted Philadelphia 26 strain of measles was grown in suckling hamsters inoculated intracerebrally, Coxsackie B3 was propagated in HeLa cell tissue cultures, and influenza A strain PR8 was prepared in lungs of mice.

Influenza neurotoxin. Influenza A strain WS was propagated in the allantoic sac of 10-day-old embryonated hen's eggs. The virus was concentrated 10-fold by high speed

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† Obtained from Antara Chemical Co., New York.

centrifugation. Titrations for neurotoxicity were carried out in weanling white mice weighing no more than 10 g each. Groups of 10 mice were inoculated intracerebrally with 0.03 ml amounts of serial decimal dilutions of concentrate in phosphate buffered saline solution. Twice daily, on days 1, 2, 3, and 4 post-inoculation, each mouse was suspended by the tail and twirled vigorously 20 to 30 times. Mice which developed tonic or clonic convulsions, whether or not resulting in death, were considered positive. Mice which survived were found to be refractory to immediate reinduction of convulsions. *Viral infectivity titrations* were carried out by ordinary procedures employing serial 10-fold dilutions of virus and the host, age, dose and volume specifications given in Table II. Six animals were employed per dilution. For the experiment summarized in Table IV, the brains of mice in each group were removed, pooled, ground with phosphate buffered saline solution to give a 5.0% suspension, and clarified by centrifugation at low speed. Egg infectivity titrations of mouse brain suspensions were carried out in allantoic cavities of 9- to 11-day-old embryonated hen's eggs. Each egg was tested for presence of hemagglutinin for chicken erythrocytes following incubation for 2 days at 36°C. For titrating *hemagglutinin* in mouse brain, samples were treated with an equal volume of cholera filtrate for 1 hour at 37°C to destroy non-specific inhibitors. Enzymic activity was stopped by adding sodium citrate in a final concentration of 0.7%. Hemagglutination titrations were carried out by ordinary procedures employing chicken erythrocytes and titer recorded as highest initial dilution of brain suspension which caused partial or complete hemagglutination.

Results. Effect of lipid A on viral neurotoxicity. Groups of 10 weanling white mice were injected intracerebrally into 1 hemisphere with *E. coli* endotoxin, lipid A or *V. cholera* filtrate as shown in Table I. Twenty-four hours later, the mice were challenged intracerebrally in the opposite hemisphere with five 50% toxic convulsive doses of neurotoxic influenza WS virus concentrate. It is apparent that all 3 preparations were highly active in inducing resistance to the neurotoxin.

TABLE I. Effect of Cerebral Injection of Lipid A and of Microbial Endotoxins on Neurotoxicity of Concentrated Influenza A (WS strain) Virus in Mice.

Material injected		Result of challenge with five 50% toxic convulsive doses of influenza virus	
Kind	Dose*	No. convulsing / Total	Convulsing %
E. coli			
Endotoxin	1 μg	0/10	0
Lipid A	3 "	1/10	10
V. cholera			
"Filtrate"	.03 ml	1/10	10
Control (Saline)	.03 "	10/10	100

* Single dose administered intracerebrally in 0.03 ml given 24 hours prior to challenge.

Effect of lipid A on viral infectivity. Viral infectivity titrations (lethal endpoint) were carried out in mice or hamsters which were prepared with lipid A or held untreated. Lipid A was highly active against PVM virus when the lipid was given by the nasal route; lipid given subcutaneously was without effect, (Table II). There was a pattern of slight protective effect against influenza A PR8 when lipid A was given subcutaneously, against neurotropic influenza NWS and herpes simplex when given intracerebrally, and against encephalomyocarditis virus following inoculation by the intraperitoneal route. Following administration of lipid by other routes or against other viruses, activity was either absent or was of such low order as to be of questionable significance.

Of all viruses shown in Table II, lipid A was most active against PVM. Further experiments were carried out to determine whether the lipid would be active if given following PVM virus. It is seen in Table III that the substance was as active when given 24 hours following virus as when given before virus. A favorable effect was obtained even when its administration was delayed for 3 days post virus inoculation.

Lipid A treatment reduced the infectivity titer in mice for encephalomyocarditis virus by 0.61 log (Table II). This suppression was reflected also in delay in time of death of mice infected with the virus. In a separate experiment not shown in the Table, mice were each

TABLE II. Comparison, Infectivity Titers, of Several Viruses of Lipid A-Treated and Untreated Mice and Hamsters.

Challenge				Regimen – Lipid A		Results		
						Titer LD_{50} (Neg. Log_{10})		Survival Index
Virus	Route	Volumes ml	No. doses*	μg/dose	Route	Treated	Control	LD_{50}
PVM	Intranasal	.05	2	50	Intranasal	<1.0	3.0	>2.0
	"	"	1	150	Subcut.	3.50	3.50	0
Influenza A-PR8	"	"	1	250	"	5.41	6.06	.65
NWS	Intracereb.	.03	1	9	Intracereb.	5.38	6.16	.78
"	"	"	5	"	"	5.62	"	.54
"	"	"	1	150	Intraperit.	6.25	"	–.09
"	"	"	5	"	"	5.70	"	.46
Herpes simplex	"	"	1	9	Intracereb.	3.00	3.60	.60
Encephalomyocarditis (Col. SK)	Subcut.	.50	1	250	Intraperit.	6.38	6.99	.61
Poliovirus II (Lansing)	Intracereb.	.03	1	9	Intracereb.	4.50	4.50	.00
Coxsackie B3	Intraperit.	.2	1	60	Intraperit.	4.49	4.50	.01
Measles	Intracereb.	.02	1	6	Intracereb.	3.80	4.17	.37

* Lipid A was given 24 hours, pre-challenge with virus; for PVM, lipid was given at 24 and 48 hours, pre-challenge.

Measles and Coxsackie viruses were tested in suckling hamsters, influenza A-NWS in weanling mice, and all others in 4-wk-old mice.

given a single 250 μg intraperitoneal dose of lipid A 24 hours prior to challenge with 3 or 30 LD_{50} of encephalomyocarditis (Col. SK) virus by the subcutaneous route. Only 1 of 6 treated mice died following inoculation with 3 LD_{50} of encephalomyocarditis virus whereas 5 of 6 control animals died. The 5 control animals which died lived, on the average, for 5.5 days while the one treated animal which died survived for 9.5 days; the remainder lived for the full 14 days' observation period. Similar but less marked extension of life span (6.4 days for treated and 3.9 days for controls) was afforded by lipid A given prior to 30 LD_{50} of encephalomyocarditis virus.

A single dose of 0.9 μg of lipid A per mouse effected (Table II) a reduction of 0.78 LD_{50} in infectivity titer of neurotropic NWS virus in mice inoculated by the cerebral route. This suppression of lethal endpoint was not, however, accompanied by any demonstrable suppression of viral proliferation in mouse brain. Thus, as shown in Table IV, the progress of increase in the infectivity and hemagglutinin titers of brains from treated and untreated mice were essentially the same, within the 5-day period of observation.

TABLE III. Influence of Time of Administration of Lipid A on PVM Infection in Mice.

Lipid A regimen			Results		
			Titer LD_{50} (neg. log_{10})		Survival index
Time, hr	Dose, μg	Route	Treated	Control	LD_{50}
Pre-challenge					
24	15	Intranasal	2.00	3.60	1.60
Post-challenge					
24	15	"	1.75	3.60	1.85
48	"	"	1.85	—	1.75
72	"	"	2.50	—	1.10

Discussion. The present studies showed that Westphal's lipid A, like whole bacterial endotoxin(12-15), causes a sparing effect in mice and hamsters against the neurotoxicity

TABLE IV. Effect of Lipid A Given Intracerebrally on Proliferation of Neurotropic (NWS) Influenza Virus in Weanling Mice Inoculated by the Intracerebral Route.

	Titers of suspensions of infected mouse brains			
	EID_{50} (neg. log_{10})		Hemagglutinin	
Time (hr) post-infect.	Treated	Control	Treated	Control
0 hr	<1.0	<1.0	<1:1	<1:1
2 "	"	"	<1:1	<1:1
4 "	"	"	1:8	1:8
6 "	"	"	1:16	1:16
8 "	"	"	1:16	1:16
1 day	"	"	1:64	1:64
2 "	<2.0	<2.0	1:64	1:64
3 "	"	"	1:64	1:64
4 "	4.60	4.80	1:128	1:128
5 "	5.40	5.00	1:128	1:128

Dose = 9 μ lipid A in 0.03 ml given intracer. 24 hr prior to virus challenge.

of concentrated influenza A virus and against the clinical outcome of infection with certain animal viruses. These findings are consistent with those reported earlier for other host resistance factors. The latter include whole bacteria, *e.g.*, typhoid bacilli(13), cholera filtrate(9,10), Xerosin, an extract of *Achromobacter xerosis*(8,11), bacterial polysaccharides of varying degree of purity(20,21), and brain ganglioside, a macromolecular glycolipid(22). The activities of whole bacteria, cholera filtrate, Xerosin, and certain of the less purified bacterial polysaccharides might well be explained on the basis of their probable content of bacterial endotoxin. The activity of lipid A against viruses cannot be definitely ascribed to lipid because of possible microcontamination with chemically or serologically undetectable amounts of residual polysaccharide. The markedly reduced activity of lipid A compared with parent endotoxin(5,17) and the considerable activity of highly purified polysaccharide alone, such as that of the Friedlander bacillus(20,21), favor the role of polysaccharide; certainly, polysaccharide is a common denominator in all the active substances. This does not exclude the possibility, however, that both lipid and polysaccharide factors as well as other possible contaminating substances may be active in inducing host resistance.

The mechanism for the action of lipid A and endotoxin in inducing resistance to viruses is unknown but may be related to the enhanced functional capacity of the reticuloendothelial system with more rapid clearance of particles(4). These substances also induce measurable alteration in metabolism(23) of treated cells which may, in turn, be less favorable to demonstration of viral effects. In most instances, in the present study, the activity of lipid A was greatest when given into the same site as the virus as, *e.g.*, the cerebral and nasal routes. In certain instances, activity was demonstrated following subcutaneous or intraperitoneal administration, but with greatly increased dosage. Most striking was the action against PVM virus, even when the lipid was administered as late as 72 hours after the virus.

Summary. Westphal's lipid A assayed in mice and hamsters enhanced non-specific host resistance against the activities of viruses such as was demonstrated earlier for bacterial endotoxins and for other materials of microbial origin. Lipid A, given intracerebrally into mice, suppressed neurotoxicity of concentrated influenza A virus and reduced the activities of neurotropic influenza (NWS) and herpes simplex viruses as measured by survival and by survival time. Given subcutaneously, it was active against influenza A virus and, following intraperitoneal injection, was active against encephalomyocarditis virus but showed slight if any activity against measles in hamsters or neurotropic (NWS) influenza virus in mice. It was inactive against Lansing type II poliovirus and Coxsackie B3 virus when given intracerebrally and intraperitoneally, respectively. Greatest activity was in the suppression of PVM virus, even when administered up to 72 hours following viral infection. The lipid A did not appear to suppress proliferation of neurotropic NWS influenza virus even though it did reduce the clinical consequence of such infection at low virus dose. Lipid A is discussed against the background of other host resistance factors.

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Electron Microscopic Studies of Rat Leukemia Induced with Mouse Leukemia Virus.* (27564)

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The mouse leukemia virus, initially isolated from spontaneous Ak mouse leukemia(1), and subsequently passed serially through newborn mice(2), proved to be pathogenic not only for mice but also for rats(3). Electron microscopic studies of the mouse leukemia virus were carried out at first using Ak or C58 mice which developed spontaneous leukemia(4,5). Ultrathin sections prepared from leukemic organs of such mice revealed in the cytoplasm or intercellular spaces of leukemic cells the presence of spherical particles varying in size from 750 to 1200 Å in diameter. In subsequent studies similar particles were found in ultrathin sections of leukemic organs of C3H mice with virus-induced leukemia(4,6). Essentially, therefore, there was no difference between particles found in animals with "spontaneous" leukemia and those in which leukemia developed following inoculation of the virus.

Experiments here reported were carried out with the purpose of studying in the electron microscope ultrathin sections of organs from leukemic rats, in which the disease was induced with the mouse leukemia virus.

Materials and methods. Suckling, less than 5 days old, Sprague-Dawley or Osborne-Mendel rats, were inoculated i.p. (0.5 ml each) with the passage A(3) mouse leukemia virus (Gross). The filtrates used for inoculation were prepared from either organs of C3H mice with virus-induced leukemia or from organs of leukemic rats in which the disease was induced with the mouse leukemia virus.

Eleven leukemic rats (3 Sprague-Dawley, and 8 Osborne-Mendel) about 2½ to 4 months old, and 12 normal, healthy control rats (8 Sprague-Dawley and 4 Osborne-Mendel) about 2 to 9 months old, were used as donors for the preparation of ultrathin sections. The rats were sacrificed by chloroform inhalation. Fragments of bone marrow, spleen, mesenteric, peripheral lymph nodes and mediastinal tumor were removed promptly and sliced into small pieces in cold phosphate buffered 2% osmic acid. After fixation for 2 hours in the cold (10°C), the tissues were brought through a series of graded

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