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Distribution and Effects of Bacterial Endotoxin

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INTRODUCTION

This study was undertaken to determine the distribution and effects of bacterial endotoxin. Two, distinctly different approaches have been used in this investigation. Initially, Pseudomonas, Bacteroides, and Listeria have been tested for endotoxic activity using the vincristine-treated mouse as one assay system. Another assay used to detect endotoxic activity was the ability to evoke resistance to a lethal dose of endotoxin after pretreatment with these three genera of bacteria. Subsequently the effect of endotoxin and endotoxin-like materials on the course of the disease that developed after infection of mice with Naegleria fowleri was determined.

A synergistic interaction has been demonstrated with bacterial endotoxin and a variety of antineoplastic agents resulting in lethality for mice. Because the enhanced toxicity of combinations of drug and endotoxin observed in mice may also occur in man, it is important for clinicians to be aware of endotoxic activity of bacteria in managing those patients undergoing long hospitalization and lengthy drug usage. For the patient with severe burn, a malignant disease or recovering from radical surgery the prospect of bacterial infection constitutes a real hazard. Many of the antineoplastic agents are immunosuppressive which also weakens host defenses and increases the chance of opportunistic infection (Rose, 1973).

Bacteroides, Pseudomonas, and Listeria were chosen to be assayed for endotoxic activity because the organisms are easily isolated from man or the environment and are not usually pathogenic except in the compromised host. The presence of endotoxin in Pseudomonas is well documented

(Hancock et al., 1970, Sadoff, 1974, and Chester et al., 1972) but literature concerning characterization of endotoxin in Bacteroides and Listeria is sparse.

According to Sonnenwirth et al. (1972) bacteremia due to Bacteroides is not uncommon and its frequency has increased more than 10 fold over the last decade. The role of endotoxin in anaerobic gram-negative bacteremia remains to be clarified especially as only a few Bacteroides species have been examined in regard to endotoxin content. It is not known whether the lipopolysaccharide (LPS) of all gram-negative, anaerobic, nonspore-forming rods contain ketodeoxyoctonate. Detection of endotoxin in plasma in patients with sepsis caused by gram-negative anaerobes by the Limulus assay has not been reported (Sonnenwirth et al., 1972).

Sonnenwirth et al. (1972) showed with a modified Limulus assay that the LPS extracted from B. fragilis and B. melaninogenicus yielded positive assays in vitro at concentrations of 10^{-2} to 10^{-6} mg/ml. Ketodeoxyoctonate (KDO) determinations demonstrated that B. fragilis and B. melaninogenicus LPS contained 0.03558 μ moles KDO/mg and 0.0332 μ moles KDO/ mg respectively (Sonnenwirth et al., 1972); these values are about 10 fold less than those for Salmonella typhimurium (Osborn et al., 1972).

Listeria monocytogenes differs from the two organisms discussed previously in that it is a gram-positive bacterium. Extracts from the cell wall of L. monocytogenes have been reported to be mitogenic (Cohen et al., 1975, Màra et al., 1974). Stanley (1949) isolated a factor from L. monocytogenes called monocyte producing agent (MPA) that causes a monocytosis in white mice and is associated with short term lymphopenia. An endotoxin-like substance isolated from L. monocytogenes, factor E_i produces a monocytosis also. This monocytosis is associated with a

leukocytosis. The monocytosis produced by factor E_i is potentiated by the lipid moiety of E_i (Patočka et al., 1974).

According to Cohen et al. (1975), a crude cell wall rich fraction of L. monocytogenes contains a substance or substances that are mitogenic for B cells in mice (Cohen, 1975). Patočka et al. (1974) found factor E_i isolated from L. monocytogenes when used for both sensitizing and reacting factors failed to give a Shwartzman reaction. However, when factor E_i was used for sensitization followed by E. coli LPS as the reacting factor a marked Shwartzman reaction occurred. Also, E_i factor (4.5 mg/kg) given iv to rabbits was pyrogenic. Factor E_i is antigenic and in conventional and inbred mice no lethal effect was observed. However, mice administered a sublethal dose of actinomycin D and factor E_i died within 7 days. Patočka et al. (1974) also observed that the toxic effect of actinomycin D in E_i treated mouse can be prevented by anti-E_i hyperimmune serum (Patocka et al., 1974).

Srivastava and Siddique (1975) found that classic gram-negative bacterial endotoxin-like biologic material from L. monocytogenes produced toxicosis in rabbits, mice, and dogs with signs and pathologic characteristics of endotoxic shock. The toxic component was found to have the following endotoxin-like properties; pyrogenicity in rabbits, edema and erythema in rabbit skin, lethality in chicken embryos and active immunity in mice (Srivastava and Siddique, 1975).

According to Rose (1973) and Bradley et al. (1975) vincristine and mithramycin produced marked potentiation of endotoxin lethality in mice (Rose, 1973, Bradley et al., 1975). For this reason the mithramycin-treated mouse and the vincristine-treated mouse were used to assay for endotoxic activity.

Mithramycin is a potent inhibitor of ribonucleic acid (RNA) synthesis. According to Dawson (1972) this compound binds to deoxyribonucleic acid (DNA) and so inhibits DNA-dependent RNA synthesis (Dawson, 1972).

When sublethal doses of endotoxin and mithramycin were administered simultaneously a marked increase in lethality in mice was observed. The mean lethal dose on day 4 was 1.7 mg/kg. The mean lethal dose on day 4 for endotoxin was 11.2 mg/kg. Mithramycin in combination with 2 mg/kg endotoxin resulted in a 19 fold reduction in the mean lethal dose for the drug on day 4. On the other hand, endotoxin with 0.25 mg/kg mithramycin resulted in a 100 fold reduction in the mean lethal dose of endotoxin on day 5. The slopes of the dose response curve of the combination were parallel to the slopes of the dose response curves of mithramycin alone and endotoxin alone indicating that the lethal action of the combination did not involve a new toxic mechanism. The isobologram based upon 50% lethal doses of combinations of mithramycin and endotoxin was markedly synergistic. Mice usually died within 24 to 48 hours after administration of the combination of mithramycin and endotoxin. Mice pretreated with endotoxin were significantly protected from lethal action of the combination. Therefore the mechanism of the synergy appears to be drug induced hyperreactivity to endotoxicity (Bradley et al., 1975). According to Bradley et al. (1975) the mithramycin treated mouse is a sensitive indicator for endotoxic acitvity in complex materials. Specificity of the lethal effect can be assessed by rendering the mice resistant to endotoxin by pretreatment (Bradley et al., 1975).

Vincristine (VNC) is an alkaloid from the plant Vinca rosea and it inhibits mitosis. This antineoplastic agent when given in combination with endotoxin exhibits a marked increase in lethality for mice. One or

4 mg/kg VNC resulted in a significant 13.1 and 1076 fold enhancement in lethality respectively (Rose et al., 1972).

According to Rose (1973), S. typhosa endotoxin potentiated VNC toxicity in mice. The LD_{50} value with respect to VNC 3 days following administration was reduced from 8.0 mg/kg when administered alone to 1.2 mg/kg, 0.95 mg/kg and 0.54 mg/kg when given in combination with 1 mg/kg, 2 mg/kg, and 4 mg/kg S. typhosa endotoxin respectively. Potentiation ratios were 6.7, 8.4, and 14.8 for VNC respectively with doses of endotoxin of 1 mg/kg, 2 mg/kg and 4 mg/kg. The slopes of these dose response curves were parallel to the slope of the dose response curve for VNC alone. This was one of the most potent enhancements of drug lethality described by Rose (1973).

Since the slopes of the dose response curves for the combination of VNC and LPS are parallel to the slopes of the dose response curve for each agent alone, one cannot attribute lethality to drug toxicity to exclusion of the endotoxin toxicity. Mice pretreated with multiple doses of S. typhosa LPS successfully reduced the enhanced lethality with VNC-LPS challenge. However, mice pretreated with LPS and challenged with 5 mg VNC/kg only had a significantly higher mortality than untreated mice receiving the same challenge dose of VNC. From the above results it appears that the role of VNC involves an enhanced reaction of the host to endotoxin. It is the availability of endotoxin for potentiating VNC lethality that is substantially reduced in an endotoxin pretreated animal because of more rapid clearance or detoxification of the toxin (Rose, 1973).

Rose (1973) observed that administration of VNC to rhesus monkeys predisposed the animals to P. aeruginosa sepsis and death. Eleven out of 14 monkeys pretreated 4 days earlier with 2.5 mg VNC and given intravenous inoculation of 5×10^{10} Pseudomonas organisms died. None of the

monkeys given Pseudomonas alone died. Saslaw suggested that VNC induced leukopenia was causally related to Pseudomonas sepsis and death in monkeys. According to Rose (1973), Pseudomonas endotoxin may have interacted with VNC to augment the observed lethality (Rose, 1973).

Endotoxin has many other effects on the host other than those mentioned previously. Another interesting effect of endotoxin is its ability to enhance or inhibit the pathogenicity of infection depending on the infecting microorganism, dose and route of injection of endotoxin, and the interval between administration of toxin and initiation of infection (Cluff, 1971).

Primary amebic meningoencephalitis was chosen as the experimental model to see if endotoxin and endotoxin-like materials could alter the course of infection. The etiological agent of primary amebic meningoencephalitis is Naegleria fowleri (Duma et al., 1969). Outbreaks of primary amebic meningoencephalitis have been reported in Australia, Czechoslovakia, and the United States. According to Duma et al. (1969) clinicopathologically the disease presents a unique picture. Those affected have been almost always healthy children or young adults with a history of swimming in fresh or brackish water during the week before onset of symptoms. Spinal fluids have been typical of acute purulent meningitis. The course has been one of rapid deterioration and despite intensive therapy, death has occurred in three to five days (Duma et al., 1969).

The mouse as an experimental model for studying primary amebic meningoencephalitis is uniquely appropriate since the existence of this disease in man was suggested following the discovery of fatal meningoencephalitis in mice after intranasal inoculation of a species of Acanthamoeba. When primary amebic meningoencephalitis was subsequently

reported in humans the remarkable similarity of the disease occurring in man to that produced experimentally was promptly recognized (Culbertson et al., 1958).

The basic features of the disease in man have been noted in experimental infections in the mouse namely the same incubation period and portal of entry, residence of amebae in the olfactory mucosa with invasion and migration through submucosal structures and into nerve plexuses, passage of amebae through pores of the cribriform plate and into the subarachnoid space, subsequent invasion of olfactory bulbs and lobes with spread to more distant areas of the brain (Martinez et al., 1973).

Treatment of naeglerial infections has been disappointing. Initially treatment consisted of antibacterial agents and antiprotozoal drugs with no success. These drugs are probably ineffective because they fail to cross the blood brain barrier. Amphotericin B has been the only drug that showed promising effects on naeglerial infection (Carter, 1972). The literature states that a 14 year old male from Australia was cured of primary amebic meningoencephalitis after intense treatment with the antifungal drug (Shumaker et al., 1971).

Endotoxic activity of several strains of bacteria were assayed in this study using the vincristine treated mouse and increased resistance of mice to a lethal dose of endotoxin because of pretreatment. Also the ability of endotoxin and endotoxin-like materials to protect mice from a fatal outcome of primary amebic meningoencephalitis was determined.

All of the strains of Pseudomonas proved to have endotoxic activity by the two assay systems used. Some of the strains of Bacteroides and Listeria had endotoxic activity by the above assays. It was also found that the course of the naeglerial infection could be altered initially by the pretreatment of mice with endotoxin. Endotoxin-like materials,

however, afforded no increased resistance of mice to primary amebic meningoencephalitis.

Materials and Methods

Male BALB/c and male DUB/ICR mice weighing 20-25 g were used in all experiments. Mice were obtained from Flow Research Animals, Inc. Dublin, Virginia. They were allowed to adjust to their new environment for at least 1 week prior to experimentation. The mice were given free access to food (Purina Lab Chow, Purina Ralston Corp., St. Louis, Mo.) and water.

All drug solutions were prepared so that 0.01 ml/g of mouse weight would provide the desired dose. All drugs and LPS injections were given simultaneously by the intraperitoneal route unless otherwise specified.

The folowing drugs were dissolved in 0.15 M NaCl: vincristine sulfate (NSC-67574; Eli Lilly and Company, Indianapolis, Ind.), and mithramycin (NSC-24559; Charles Pfizer and Company, Clifton, N. J.).

Escherichia coli 0127:B8 LPS, E. coli 026:B6 LPS, E. coli 0111:B4 LPS (all Boivin preparations of LPS, Difco Laboratories, Detroit, Mich.) and E. coli 026:B6 LPS, and Salmonella typhosa 0901 LPS (Westphal preparations of LPS, Difco) were suspended in 0.15 M NaCl. E. coli 026:B6 LPS (Boivin preparation, Difco) was used unless otherwise specified.

Bacteroides strains were kindly supplied by P. B. Hylemon (Department of Microbiology, Virginia Commonwealth University). The Bacteroides cultures were grown overnight at 37° C under anaerobic conditions in medium containing 1 g peptone, 1 g yeast extract, 0.5 g glucose, 4 ml salt solution, and 100 µg hemin per 100 ml distilled H_2O.

The salts solution contained 0.02 g $CaCl_2$, 0.02 g $MgSO_4$, 0.1 g K_2HPO_4, 0.1 g KH_2PO_4, 1 g NaCl per 100 ml distilled H_2O. After autoclaving 0.1 g L-cysteine and 0.4 g Na_2CO_3 per 100 ml of medium were added and the pH adjusted to 7.0 with sterile 6 N HCl.

The Listeria strains were kindly supplied by H. J. Welshimer (Department of Microbiology, Virginia Commonwealth University). The Listeria cultures were grown overnight at 37° C in a medium consisting of 20 g of tryptose (Difco), 2 g of NaCl, 3.5 g Na_2HPO_4 and 2 g glucose per liter.

The Pseudomonas strains were kindly supplied by P. V. Phibbs (Department of Microbiology, Virginia Commonwealth University). The Pseudomonas cultures were grown overnight in a controlled environment incubator shaker (New Brunswick Scientific, New Brunswick, N. J.) at 37° C in nutrient broth consisting of 1 g peptone (Difco), 0.3 g beef extract (Difco) and 0.5 g NaCl per 100 ml distilled H_2O.

All bacterial cells were harvested by centrifugation in a refrigerated centrifuge at 9000 X G for 15 min and suspended in sterile 0.15 M NaCl. The turbidity at 420 nm was measured on a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, N. Y.). Listeria cells were killed prior to harvesting by heating in a 70° C water bath for 1 hr. Pseudomonas cells were harvested, collected in saline and killed by heating in a 70° C water bath for 1hr.

The protozoan culture Naegleria fowleri (Lee strain) was kindly supplied by D. T. John (Department of Microbiology, Virginia Commonwealth University). Naegleria was grown in Nelson's medium consisting of Page saline (0.012 g NaCl, 400 µg $MgSO_4 \cdot 7H_2O$, 400 µg $CaCl_2 \cdot 2H_2O$, 0.0142 g Na_2HPO_4, 0.0136 g KH_2PO_4 per 100 ml distilled H_2O) supplemented with 0.1 g liver infusion (Oxoid, London, England), 0.1 g glucose, and 2 ml

calf serum (Grand Island Biologicals Company, Grand Island, N. Y.) per 100 ml of Page saline. Medium (25 ml) in tissue culture flasks 75 cm^2 (Falcon Plastics, Oxnard, Cal.) were inoculated and incubated 96 hr at 37^o C.

The trophozoites were harvested by centrifugation at 4000 X G for 10 min in a refrigerated centrifuge, washed with Page saline (described above), washed with 0.15 M NaCl, suspended in 0.15 M NaCl and counted using a hemocytometer. The cells were then diluted so that 0.2 ml would deliver the desired inoculum. All N. fowleri was given intravenously unless otherwise specified.

Mice surviving from naeglerial infection were divided into two groups. Half of the mice were rechallenged intravenously with N. fowleri (5.0×10^6 trophozoites/mouse). After 14 days both groups of survivors were treated with 200 mg cyclophosphamide (Mead Johnson Laboratories, Evansville, Indiana)/kg administered intraperitoneally.

Mice were also administered N. fowleri intraperitoneally and subcutaneously. After 17-20 days, the mice challenged ip were rechallenged iv with 5.0×10^6 Naegleria/mouse.

Lipid A was extracted from E. coli 0127:B8 lipopolysaccharide by a procedure adapted from the method of Galanos et al. (1969). The absence of 2 keto-3-deoxyoctonate as confirmed by the thiobarbiturate assay of Waravdekar and Saslaw (1959) was used to establish that hydrolysis was complete. Complexes of lipid A and bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) or concanavalin A (grade IV; Sigma) were prepared by methods described by Galanos et al. (1972). Immediately before use the complexes were suspended in distilled water and dispersed with sonication. Complexes of bovine serum albumin (Sigma) with myristic acid (Sigma) or N,N-dimethyl myristamide (Sigma) and com-

plexes of concanavalin A (Sigma) with beta hydroxymyristic acid were also tested for endotoxic activity. Immediately before use the complexes were suspended in distilled water and dispersed with sonication.

RESULTS

Enhanced lethality of combinations of mithramycin and LPS occurred when the two agents were administered simultaneously. LPS given intraperitoneally (ip) or intravenously (iv) rendered the mice hyperreactive to mithramycin administered ip or iv (Table 1). The ip route of administration was chosen for subsequent work because more mice were killed for a given dose by the ip route than by the iv route and because of the ease of inoculation by the ip route.

Five LPS preparations were tested for their ability to interact in vivo with mithramycin. Four of the preparations were from E. coli; two of these were prepared by the Boivin technique. The other three LPS preparations were obtained by the Westphal technique (Table 2). All five preparations given in combination with 0.5 mg mithramycin/kg resulted in enhanced lethality. LPS from E. coli 026:B6 was not as potent as LPS from E. coli 0127:B8, E. coli 0111:B4 or S. typhosa 0901. E. coli 0126:B6 LPS (B) (Table 2) was selected for subsequent experiments because of its intermediate potency and because E. coli is found as part of the normal flora in man and mouse.

Three genera of bacteria have been tested for endotoxic activity using the vincristine-treated mouse as one assay system. Another assay used to detect endotoxic activity was the ability to evoke resistance to a lethal dose of endotoxin after pretreatment with three genera of bacteria.

Table 3 shows that high doses of all Pseudomonas aeruginosa strains tested caused death in both the untreated and the vincristine-treated mouse within 24 hr. When the sample of bacteria was diluted approxi-

mately 1:4 (see Table 4) there was substantial death in all the vincristine treated animals. Only two of the strains of Pseudomonas (PPR 37 and PPR 38) when administered alone caused death in mice. Table 5 shows that all strains of P. aeruginosa afforded protection to mice challenged with a lethal dose of bacterial endotoxin. By the two assay systems used all strains of P. aeruginosa possess endotoxic activity.

Seven strains of Bacteroides were tested for endotoxic activity. All strains of Bacteroides were members of the species B. fragilis except 7989 which was a strain of B. melaninogenicus.

Four of the seven strains of Bacteroides (7989, 5482, 4245, and 2393) showed endotoxic activity in the vincristine treated mouse (Table 6). All strains of Bacteroides protected mice from subsequent challenge with a lethal dose of LPS (Table 7).

Mice treated with the following strains of Listeria monocytogenes (19303, PW, D-9, V-6, and H-3) were rendered somewhat more susceptible to a dose of 1 mg VNC/kg (Table 8). Mice challenged with five strains of Listeria monocytogenes (MCV, V-5, T_4b, FH-1, and T_3b) were not detectably sensitized to heat killed cells by 1 mg VNC/kg. Mice given a single dose of heat killed Listeria cells 4-7 days prior to challenge with 20 mg E. coli LPS/kg were protected when pretreated with 19303, PW, D-9, T_3b and H-3 but not when pretreated with MCV, V-5, T_4b, FH-1 and V-6 (Table 9).

The time of death after simultaneous administration of a bacterial suspension and 1 mg VNC/kg is shown in Figure 1. Mice administered 20 mg LPS/kg alone all died within the first 24 to 48 hr. Time of death in mice given bacterial suspensions and VNC was delayed compared to the time of death after the LPS alone.

TABLE 1

Effect of route of administration on the enhanced lethality of mithramycin and LPS[a]

Mithramycin Dose mg/kg	Route	Route for 0.1mg LPS/kg	No. of mice	% Dead by day 4
0.5	ip	--	20	0
0.5	iv	--	30	0
0.5	--	iv	20	0
0.5	--	ip	10	0
0.5	ip	ip	30	97
0.5	ip	iv	30	70
0.5	iv	ip	30	53
0.5	iv	iv	30	77
0.25	iv	--	8	0
0.25	iv	ip	30	10
0.25	ip	iv	40	10
0.25	iv	iv	30	23
0.25	ip	ip	30	41

[a]Male DUB/ICR mice were injected with mithramycin and S. typhosa 0901 (Westphal) LPS simultaneously by the routes and doses indicated.

TABLE 2

Specificity of the in vivo lethal interaction between mithramycin and bacterial LPS[a]

Mithramycin	LPS	No. of Mice	% Dead
0.5mg/kg	E. coli 0127:B8 (B) 1mg/kg	10	100
0.5mg/kg	E. coli 0127:B8 (B) 0.1mg/kg	10	100
0.25mg/kg	E. coli 0127:B8 (B) 1.0mg/kg	10	60
0.25mg/kg	E. coli 0127:B8 (B) 0.1mg/kg	10	50
0.5mg/kg	E. coli 026:B6 (B) 1.0mg/kg	10	90
0.5mg/kg	E. coli 026:B6 (W) 1mg/kg	10	80
0.5mg/kg	E. coli 026:B6 (B) 0.1mg/kg	10	60
0.5mg/kg	E. coli 026:B6 (W) 0.1mg/kg	10	70
0.25mg/kg	E. coli 026:B6 (B) 1.0mg/kg	10	40
0.25mg/kg	E. coli 026:B6 (W) 1.0mg/kg	10	0
0.25mg/kg	E. coli 026:B6 (B) 0.1mg/kg	10	0
0.25mg/kg	E. coli 026:B6 (W) 0.1mg/kg	10	0
0.5mg/kg	E. coli 0111:B4 (W) 1mg/kg	10	80
0.5mg/kg	E. coli 0111:B4 (W) 0.1mg/kg	10	100
0.25mg/kg	E. coli 0111:B4 (W) 1.0mg/kg	10	90
0.25mg/kg	E. coli 0111:B4 (W) 0.1mg/kg	10	100
0.5mg/kg	S. typhosa 0901 (W) 1mg/kg	10	100
0.5mg/kg	S. typhosa 0901 (W) 0.1mg/kg	10	100
0.25mg/kg	S. typhosa 0901 (W) 1.0mg/kg	10	100
0.25mg/kg	S. typhosa 0901 (W) 0.1mg/kg	10	100

[a]Male BALB/c mice were injected simultaneously by the ip route with mithramycin and LPS. (B) and (W) refer to Boivin and Westphal preparations respectively. A dose of 1mg E. coli 026:B6 (B)/kg killed 1 of 10 treated mice; this dose of the other five LPS preparations did not kill any of the treated mice (10 mice/group).

TABLE 3

Capability of heat-killed strains of Pseudomonas to enhance the lethal action of vincristine for mice[a]

Strain[b]	A_{420}	1mg VNC/kg	No. mice	% Dead
PPR 25	45	-	10	80
PPR 25	45	+	10	80
PPR 37	46	-	10	90
PPR 37	46	+	10	100
PPR 38	40	-	10	80
PPR 38	40	+	10	100
PPR 17	45	-	10	60
PPR 17	45	+	10	90
PPR 43	39	-	10	90
PPR 43	39	+	10	100
PPR 42	44	-	10	70
PPR 42	44	+	10	100
PPR 35	25	-	9	89
PPR 35	25	+	10	90
PPR 11	41	-	10	100
PPR 11	41	+	10	90
PPR 18	45	-	10	60
PPR 18	45	+	10	80
PPR 36	46	-	10	80
PPR 36	46	+	10	80
1mg LPS/kg	--	-	10	0
1mg LPS/kg	--	+	10	20
--	--	+	30	10

[a]DUB/ICR male mice were injected simultaneously ip with Pseudomonas (0.01 ml/g mouse; the turbidity of the suspension is indicated in the table) and 1mg VNC/kg where indicated. The percent of animals dead within 7 days is recorded.

[b]All strains of Pseudomonas were isolated from patients with cystic fibrosis.

TABLE 4

Capability of heat-killed strains of Pseudomonas aeruginosa to enhance the lethal action of vincristine for mice[a]

Strain	A_{420}	1mg VNC/kg	No. mice	% Dead
PPR 25	9	-	10	0
PPR 25	9	+	10	60[b]
PPR 37	11	-	10	20
PPR 37	11	+	10	60
PPR 38	9	-	10	10
PPR 38	9	+	10	40
PPR 17	10	-	10	0
PPR 17	10	+	10	40
PPR 43	8	-	10	0
PPR 43	8	+	10	40
PPR 42	8	-	10	0
PPR 42	8	+	10	40
PPR 35	8	-	10	0
PPR 35	8	+	10	40
PPR 11	10	-	10	0
PPR 11	10	+	10	30
PPR 18	11	-	10	0
PPR 18	11	+	10	30
PPR 36	11	-	10	0
PPR 36	11	+	10	20
1mg LPS/kg	--	-	10	0
1mg LPS/kg	--	+	10	20
--	--	+	30	10

[a]DUB/ICR male mice were injected simultaneously ip with Pseudomonas (0.01 ml/g mouse; the turbidity of the suspension is indicated in the table) and 1mg VNC/kg where indicated. The percent of animals dead within 7 days is recorded.

[b]Significant at $p<0.02$ for the VNC-treated mice in contrast to the untreated control.

Combining the results for all strains of P. aeruginosa, the VNC-treated mice are significantly more sensitive to LPS than the untreated mice at $p<0.01$.

TABLE 5

Capability of strains of *Pseudomonas* to produce increased resistance to lethal doses of bacterial endotoxin[a]

Strain	A_{420}	No. mice	% Dead
PPR 25	9	10	10
PPR 37	11	8	0
PPR 38	9	9	11
PPR 17	10	10	0
PPR 43	8	10	0
PPR 42	8	10	0
PPR 35	8	10	10
PPR 11	10	10	10
PPR 13	11	10	0
PPR 36	11	10	0
20mg LPS/kg	--	10	60

[a]DUB/ICR male mice were given a single injection ip with *Pseudomonas* (0.01 ml/g of mouse; the turbidity of the suspension is indicated in the table). About 1 week later mice were challenged with 20mg *E. coli* 026:B6 LPS (B). $p<0.01$ for all pretreated groups compared to the untreated group.

TABLE 6

Capability of strains of Bacteroides to enhance the lethal action of vincristine for mice[a]

Strain	A_{420}	1mg VNC/kg	No. mice	% Dead
7989[b]	43	-	20	0
7989[b]	43	+	20	40[d]
5482[c]	26	-	10	0
5482[c]	26	+	10	40
4245[c]	19	-	10	10
4245[c]	19	+	10	40
2393[c]	39	-	20	0
2393[c]	39	+	20	25[d]
3524[c]	36	-	10	0
3524[c]	36	+	10	0
3452-A[c]	41	-	10	0
3452-A[c]	41	+	10	0
25285[c]	38	-	10	0
25285[c]	38	+	10	0
1mg LPS/kg	--	-	10	0
1mg LPS/kg	--	+	10	20

[a]DUB/ICR male mice were injected simultaneously ip with Bacteroides (0.01 ml per 1g of mouse; the turbidity of the suspension is indicated in the table) and 1mg VNC/kg when indicated. The percent of animals dead within 7 days is recorded.

[b]B. melaninogenicus

[c]B. fragilis - Subspecies of B. fragilis are: fragilis, 25285 and 2393; vulgatus, 4245; thetaiotaomicron, 5482; ovatus, 3524; distasonis, 3452-A.

[d]Significant at $p<0.02$ for the VNC-treated mice in contrast to the untreated control.

Combining the results for all strains of B. fragilis, the VNC-treated mice are significantly more sensitive to LPS than the untreated mice at $p<0.01$.

TABLE 7

Capability of strains of Bacteroides to produce increased resistance to lethal doses of bacterial endotoxin[a]

Strain	A_{420}	No. mice	% Dead
7989	42	20	20
5482	26	10	10
4245	19	10	30
2393	39	20	5
3524	36	10	10
3452A	41	10	0
25285	38	10	10
20mg/kg LPS	--	10	60

[a]DUB/ICR male mice were given a single injection ip with Bacteroides (0.01 ml/g of mouse; the turbidity of the suspension is indicated in the table). About 1 week later mice were challenged with 20mg E. coli 026:B6 LPS (B). The specific identification of these strains is given in Table 6. $p<0.05$ for all pretreated groups compared to the untreated group.

TABLE 8

Capability of heat-killed strains of Listeria to enhance the lethal action of vincristine for mice[a]

Strain	A_{420}	1mg VNC/kg	No. mice	% Dead
MCV[b]	43	-	10	0
MCV[b]	43	+	10	0
19303[b]	40	-	10	0
19303[b]	40	+	20	15
PW[b]	36	-	10	0
PW[b]	36	+	20	45[c]
V5	42	-	10	0
V5	42	+	10	0
T_4b[b]	39	-	10	10
T_4b[b]	39	+	10	20
FH-1	40	-	10	0
FH-1	40	+	10	0
D-9	46	-	10	0
D-9	46	+	20	15
V-6	43	-	10	0
V-6	43	+	20	30
T_3b[b]	39	-	10	10
T_3b[b]	39	+	20	5
H-3	41	-	10	0
H-3	41	+	10	20
--	--	+	30	10
1mg/kg LPS	--	-	10	0
1mg/kg LPS	--	+	10	20

[a]DUB/ICR male mice were injected simultaneously ip with Listeria (0.01 mg/g mouse; the turbidity of the suspension is indicated in the table) and 1mg VNC/kg when indicated. The percent of animals dead within 7 days is recorded.

[b]Virulent strains

[c]Significant at $p<0.01$ for the VNC-treated mice in contrast to the untreated control.

Combining the results for all strains of L. monocytogenes, the VNC-treated mice are significantly more sensitive to LPS than the untreated mice at $p<0.01$.

TABLE 9

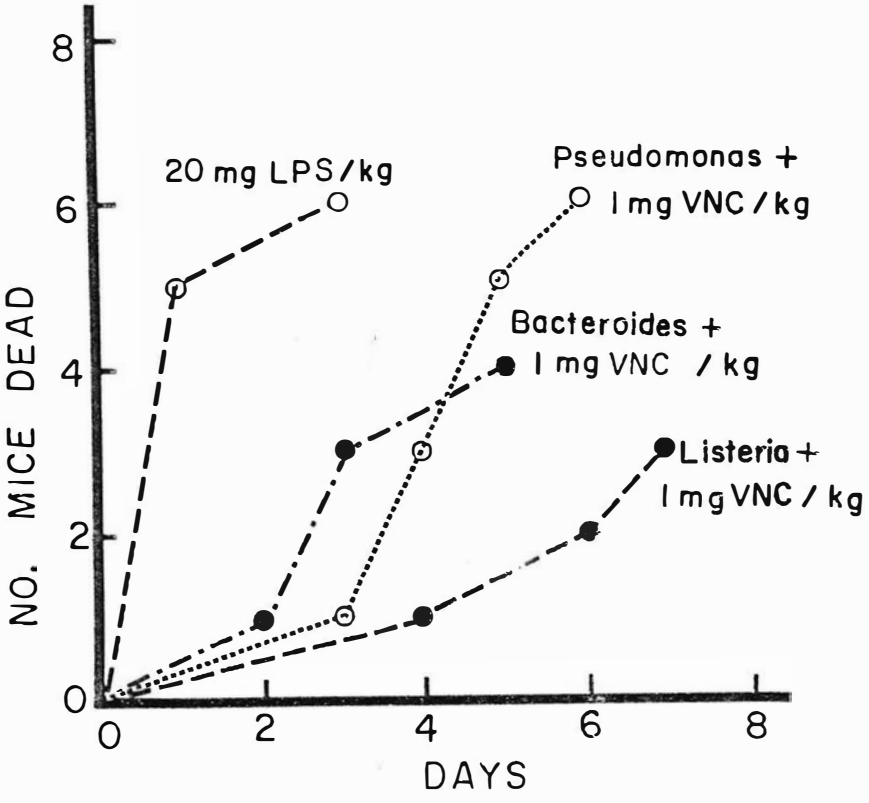
Capability of heat-killed strains of *Listeria monocytogenes* to produce increased resistance to lethal doses of bacterial endotoxin[a]

Strain	A_{420}	No. mice	% Dead
MCV	43	8	38
19303	40	18	22
PW	36	18	5
V5	42	8	38
T_4b	39	16	13
FH-1	40	8	25
D-9	46	18	11
V-6	43	18	44
T_3b	39	7	0
H-3	41	19	11
none	--	10	60

[a]DUB/ICR male mice were given a single injection of *Listeria* (0.01 ml/g mouse; the turbidity of the suspension is indicated in the table). About 1 week later, mice were challenged with 20mg *E. coli* 026:B6.

[b]Significant at $p<0.05$ for the VNC-treated mice in contrast to the untreated control.

Figure 1. Time of death of mice after simultaneous administration of bacterial suspension and 1 mg VNC/kg or 20 mg *E. coli* 026:B6 LPS/kg alone. The number of animals for this experimental series was: 10 for 20 mg LPS/kg; 10 for *Pseudomonas* + 1 mg VNC/kg; 20 for *Bacteroides* + 1 mg VNC/kg; 20 for *Listeria* + 1 mg VNC/kg.


8
6
4
2
0
NO. MICE DEAD
20 mg LPS / kg
Pseudomonas +
1 mg VNC / kg
Bacteroides +
1 mg VNC / kg
Listeria +
1 mg VNC / kg
0
2
4
6
8
DAYS

The course of infection of Naegleria fowleri seems to be dose dependent whether administered via the intraperitoneal (ip) or intravenous (iv) route. Figure 2 shows after iv administration of 1×10^7 trophozoites/mouse 100% of the mice were dead by day 10 as compared to 70% mortality on day 10 for a dose of 5.0×10^6 Naegleria/mouse and 40% mortality on day 10 for a dose of 2.5×10^6 trophozoites/mouse. All of the mice in the group receiving 5.0×10^6 trophozoites/mouse were dead by day 13.

Signs of distress such as ruffled fur and weight loss were not observed in mice infected iv with Naegleria. However, one or two days prior to death they displayed posterior paralysis with no effect on the anterior appendages. When total paralysis was seen in mice prior to death they would respond violently to stimuli by a "cork screwing" motion. Weight loss and dehydration were seen during this time. Occasionally acute deaths occurred within an hour or two of Naegleria administration. No signs of CNS involvement were seen with the acute deaths.

Figure 3 shows that mice administered 5.0×10^6 and 1.0×10^7 trophozoites by the ip route had 10% mortality by day 18 and no mortality by day 18 respectively. However, all the mice receiving 4.85×10^7 Naegleria fowleri ip were dead by day 8. Acute deaths were observed in mice inoculated with 4.85×10^7 trophozoites/mouse with no signs of central nervous system (CNS) involvement. On day 6, however, CNS involvement became apparent as is seen in mice challenged with Naegleria fowleri iv. Mice administered 5.0×10^6 Naegleria subcutaneously did not die or show signs of CNS involvement.

Endotoxin and complexes (using portions of the endotoxin molecule complexed to protein carriers) were surveyed to determine whether

some protection could be induced in mice infected with Naegleria fowleri. Figure 4 shows the results of the effect of endotoxin at a dose of 1 mg/kg 24 hr prior to injection of Naegleria. Some protection was afforded early in the infection with mice pretreated with endotoxin. Towards day 8 there is no significant difference in survival.

None of the other agents tested produced substantial protection to Naegleria infection in mice. Figure 5, 6, and 7 show survival with time of mice pretreated with lipid A complexes and the respective carriers. No significant protection was afforded.

Figure 8, 9, and 10 show survival with time of mice pretreated with dimethylmyristamide-bovine serum albumin, dimethymyristamide, beta-hydroxy-myristic acid-con A, beta-hydroxy-myristic acid, myristic-BSA, and myristic acid, and challenged with Naegleria. None of the above agents provided significant protection against the naeglerial infection.

Figure 11 shows the results of mice pretreated with lipid A and challenged with Naegleria iv. Acute deaths were seen in the mice receiving Naegleria alone. Those mice receiving lipid A seem to be protected from the early deaths seen in the mice receiving Naegleria alone. No significant protection is seen later in the course of infection.

Mice surviving a primary iv injection of Naegleria from previous experiments were held for a period of 14 days with no death. The groups of mice containing two or more survivors were divided equally and one half of the group was rechallenged with 5×10^6 Naegleria/mouse. Figure 12 shows that the control mice receiving a primary injection of Naegleria died within a period of 5-14 days as expected. The mice surviving a primary injection with Naelgeria were significantly protected from the second challenge. Only one out of seven mice administered a primary dose and then rechallenged with Naegleria died. All of the survivors

that had received a primary dose of Naegleria but were not rechallenged with Naegleria survived but one.

Ten of the 18 mice surviving the primary challenge ip with Naegleria were rechallenged with Naegleria (5 X 10^6 trophozoites/mouse iv) 20 days after primary inoculation (Figure 13). Eight of the survivors were held as uninoculated controls. No deaths occurred in the 8 mice that received a primary ip injection of Naegleria. Control mice receiving a primary injection of Naegleria iv died between the 7th and 14th day. Only one out of the 10 mice receiving a primary ip injection and then rechallenged iv with Naegleria died on day 11 post iv inoculation. Significant protection was afforded mice pretreated ip with Naegleria and then rechallenged iv (Figure 13).

In a separate experiment ten mice surviving a previous challenge of Naegleria iv were divided into two groups. Six were treated with cyclophosphamide and 4 were used as controls. None of the mice treated with cyclophosphamide died. One out of the 4 control mice was found dead probably killed by another mouse because constant fighting was observed and the dead mouse had a large open lesion on his back.

Figure 2. Time of death of mice administered varied doses of Naegleria fowleri (Lee strain) intravenously (iv). The number of animals in each experimental series was 10.

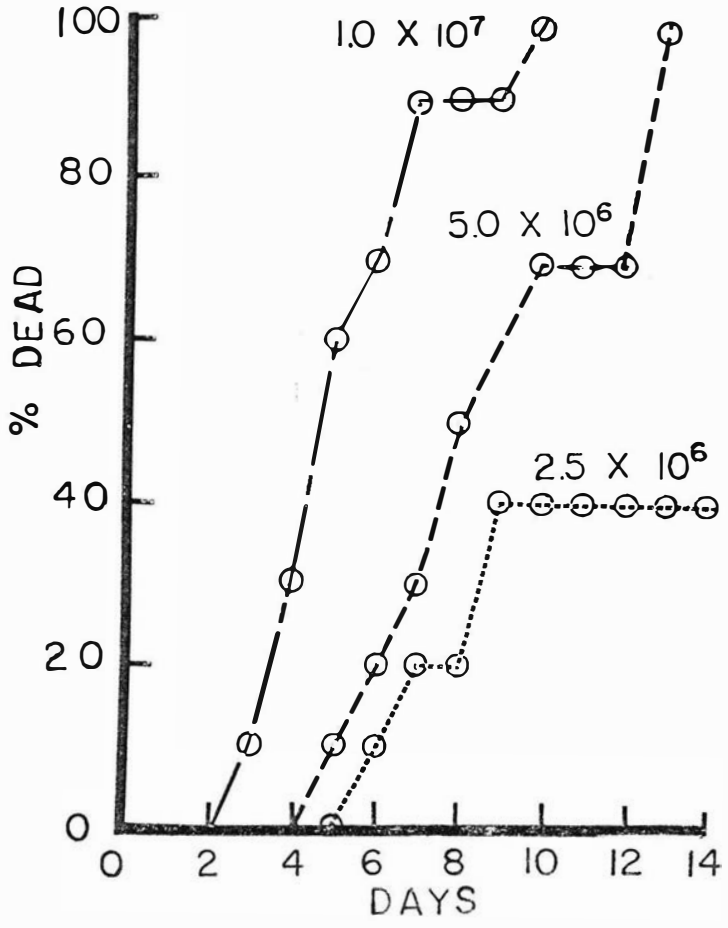

100
80
60
40
20
0
% DEAD
1.0 X 10⁷
5.0 X 10⁶
2.5 X 10⁶
0
2
4
6
8
10
12
14
DAYS

Figure 3. Time of death of mice administered varied doses of Naegleria fowleri (Lee strain) intraperitoneally (ip). Naegleria (5×10^6/mouse) was administered intravenously as a control. The number of animals in this experimental series was: 14 for a dose of 4.85×10^7 Naegleria ip; 10 for a dose of 5×10^6 Naegleria iv; 10 for a dose of 5×10^6 Naegleria ip; 8 for a dose of 1×10^7 Naegleria ip.

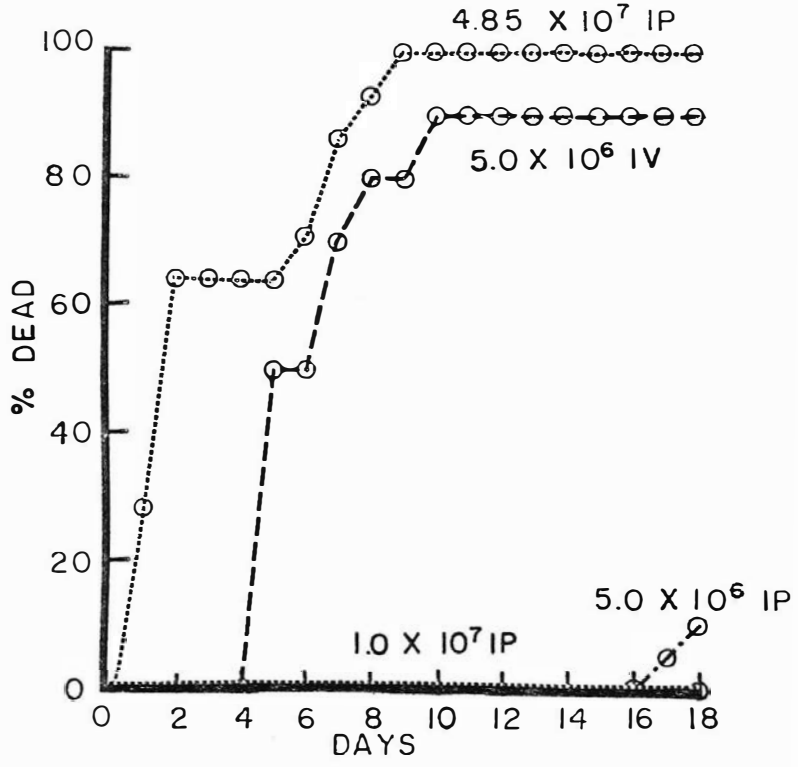

4.85 X 10⁷ IP
5.0 X 10⁶ IV
5.0 X 10⁶ IP
1.0 X 10⁷ IP
% DEAD
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
DAYS

Figure 4. The effect of endotoxin on the course of Naegleria infection in mice. Mice received 1 mg E. coli 026:B6 LPS/kg ip 24 hr prior to iv challenge with Naegleria fowleri (Lee strain). Naegleria (5×10^6/mouse) was administered iv as a control. The number of animals in this experimental series was: 39 for no LPS; 40 for 1mg LPS/kg.

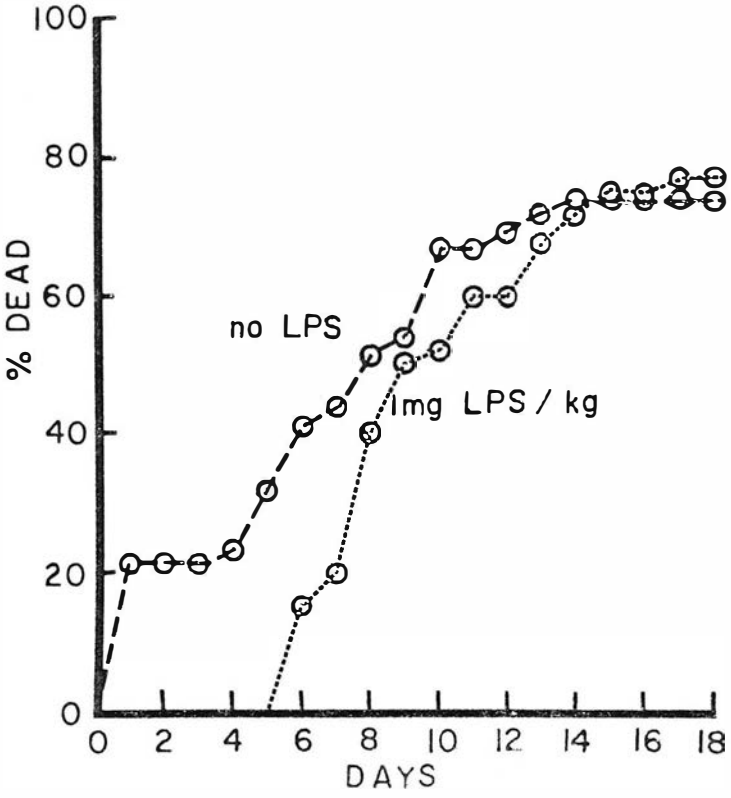
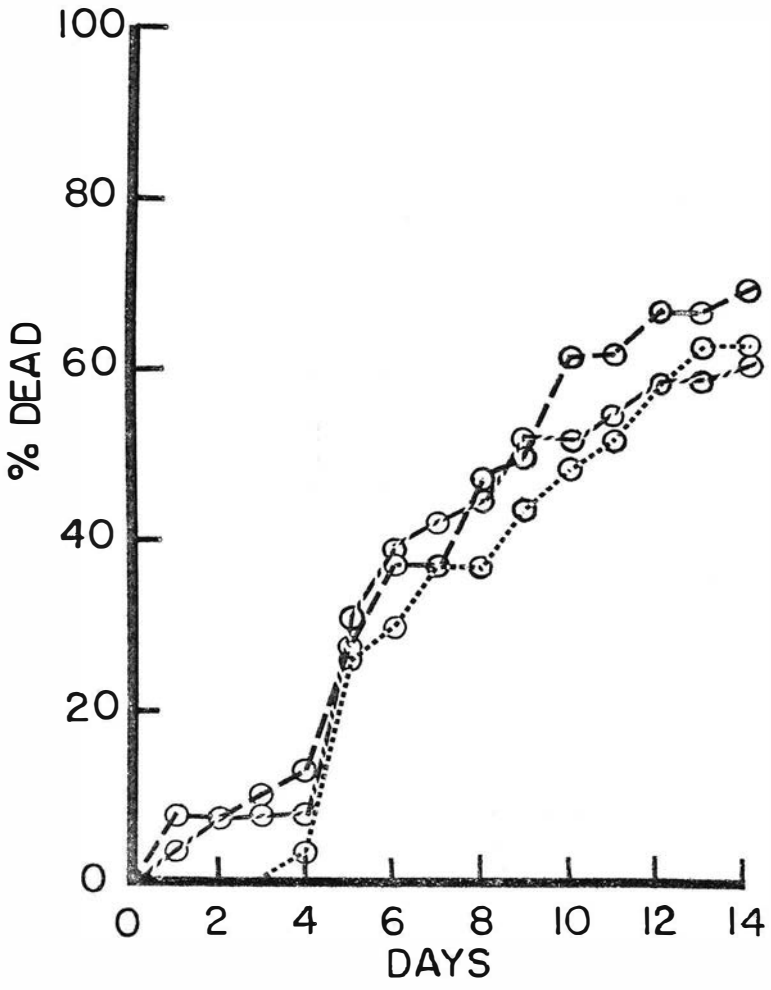

100
80
60
40
20
0
% DEAD
no LPS
1mg LPS / kg
0
2
4
6
8
10
12
14
16
18
DAYS

Figure 5. The effect of lipid A-BSA (·····⊖·····) or BSA (~~—⊖—~~) on the course of Naegleria infection in mice. Mice received 50 mg lipid A-BSA/kg or 50 mg BSA/kg ip 24 hr prior to iv challenge with Naegleria fowleri (Lee strain). Naegleria (5 X 10^6/mouse) (— ⊖ —) was administered iv as a control. The number of animals in this experimental series was: 27 for lipid A-BSA; 31 for BSA; 30 for control.


100
80
60
40
20
0
% DEAD
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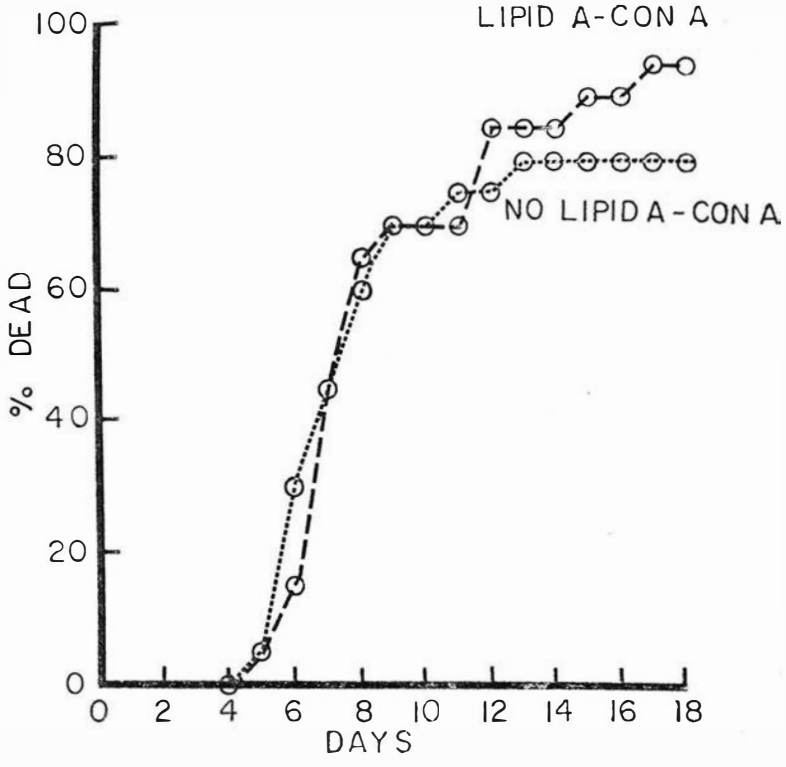
2
4
6
8
10
12
14
DAYS

Figure 6. The effect of lipid A-Con A on the course of Naegleria infection in mice. Mice received 50 mg lipid A-Con A/kg ip 24 hr prior to iv challenge with Naegleria fowleri (Lee strain). Naegleria (5×10^6/mouse) was administered iv as a control. The number of animals for each experimental series was 20.


LIPID A-CON A
NO LIPID A-CON A
% DEAD
100
80
60
40
20
0
0
2
4
6
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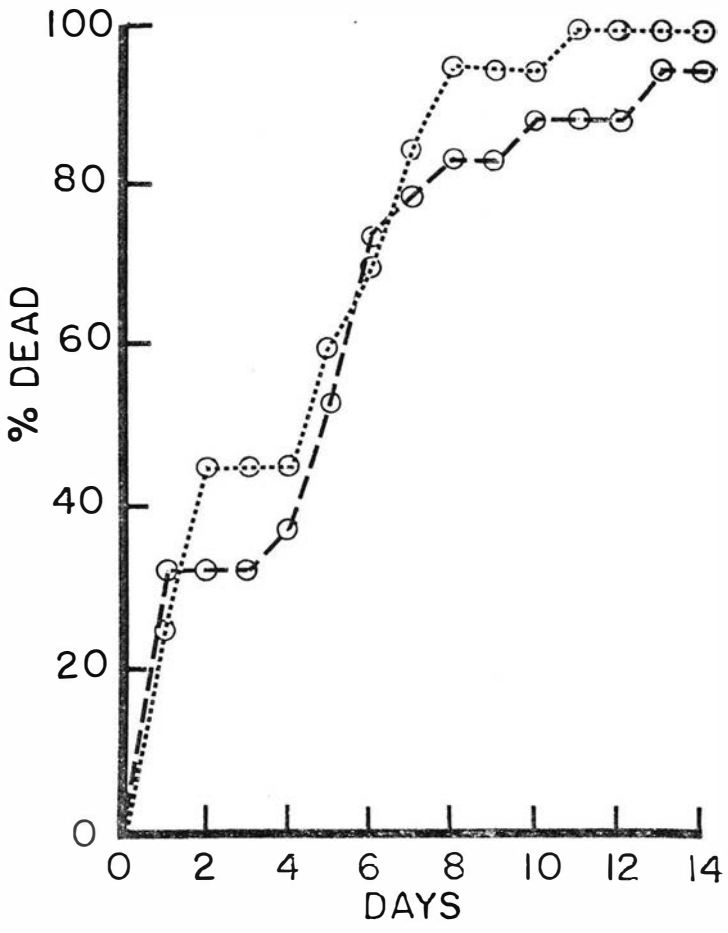
10
12
14
16
18
DAYS

Figure 7. The effect of concanavalin A on the course of Naegleria infection in mice. Mice received 50 mg concanavalin A/kg (---⊖---) ip 24 hr prior to iv challenge with Naegleria fowleri (Lee strain). Naegleria (5 X 10^6/mouse) (—⊖—) was administered iv as a control. The number of animals for this experimental series was: 20 for concanavalin A; 19 for control.


100
80
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% DEAD
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10
12
14
DAYS

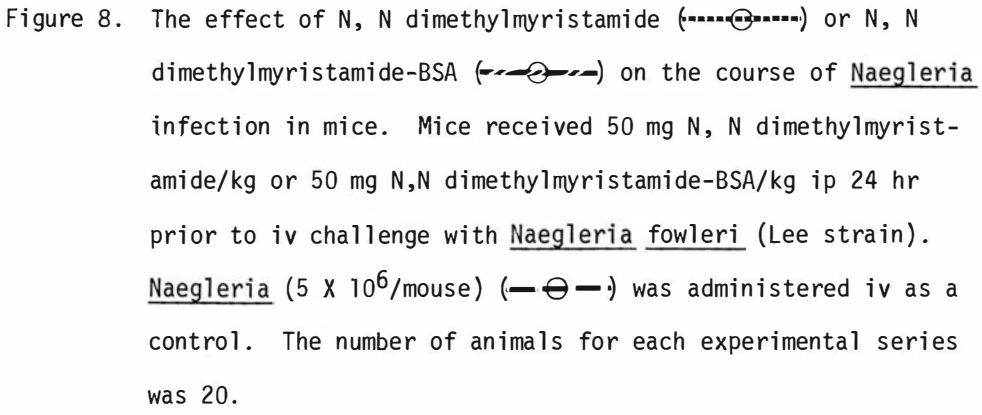
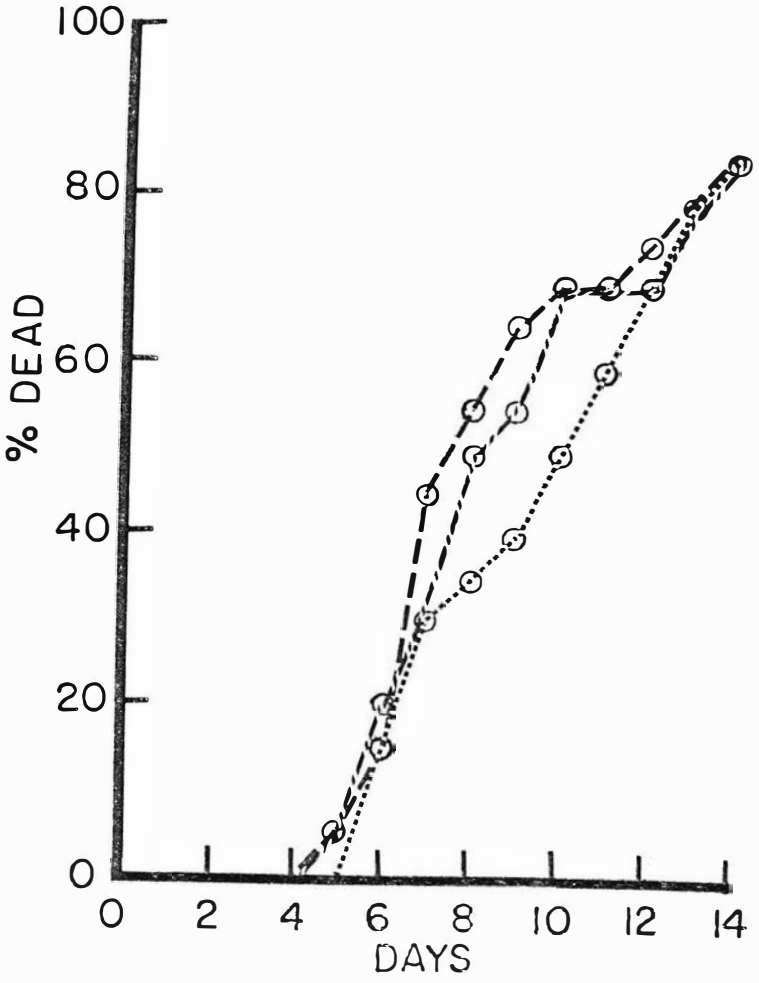


Figure 8. The effect of N, N dimethylmyristamide (·····⊖·····) or N, N dimethylmyristamide-BSA (- - -⊖- - -) on the course of Naegleria infection in mice. Mice received 50 mg N, N dimethylmyristamide/kg or 50 mg N,N dimethylmyristamide-BSA/kg ip 24 hr prior to iv challenge with Naegleria fowleri (Lee strain). Naegleria (5 X 10^6/mouse) (—⊖—) was administered iv as a control. The number of animals for each experimental series was 20.


100
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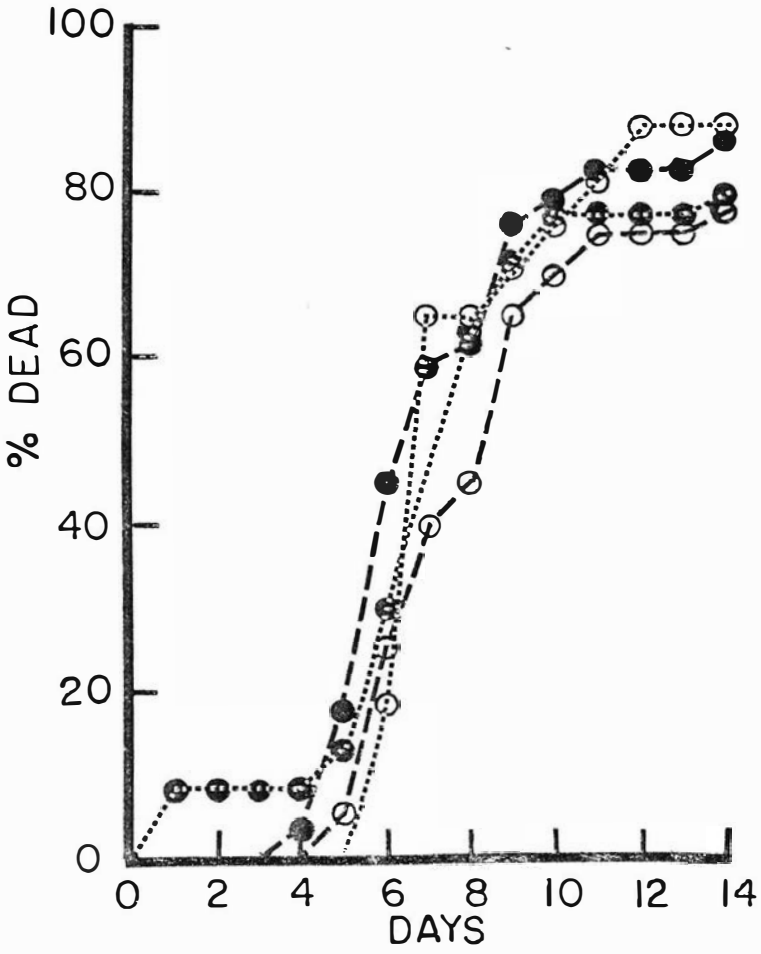
DAYS

Figure 9. The effect of beta hydroxymyristic acid (·····⊖·····) or beta hydroxymyristic-Con A (·····●·····) on the course of Naegleria infection in mice. Mice received 50 mg beta hydroxymyristic acid/kg or 50 mg beta hydroxymyristic-Con A/kg ip 24 hr prior to iv challenge with Naegleria fowleri (Lee strain). Naegleria (5×10^6/mouse) (—⊖—)(—●—) was administered iv as a control. The number of animals for this experimental series was: 30 for beta hydroxymyristic-Con A; 29 for control; 17 for beta hydroxymyristic acid; 20 for control.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DAYS

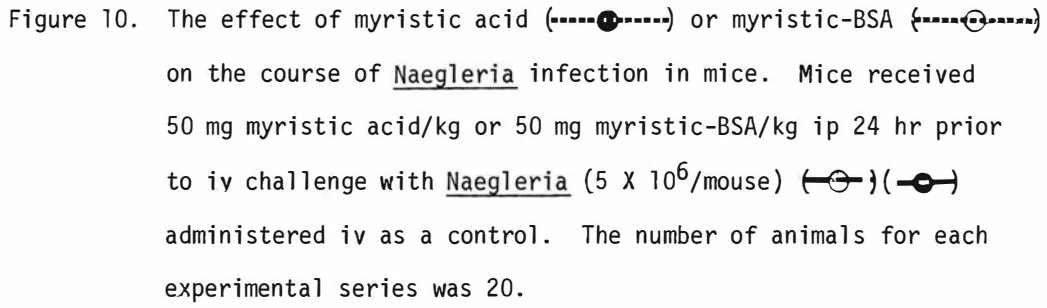
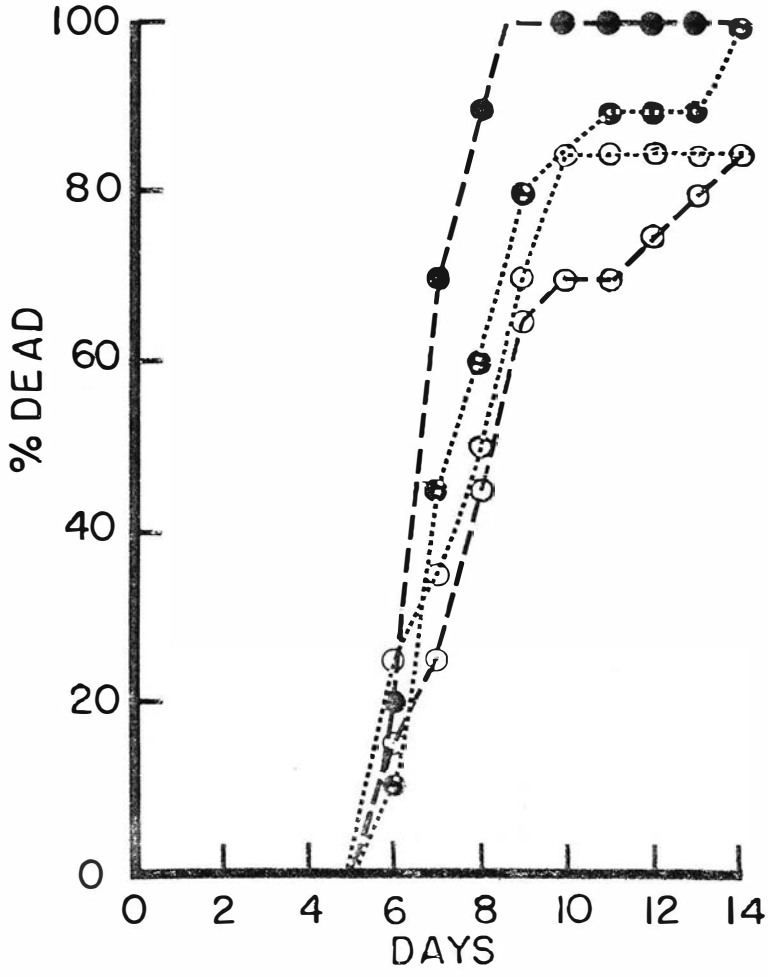


Figure 10. The effect of myristic acid (-----●-----) or myristic-BSA (-----⊖-----) on the course of Naegleria infection in mice. Mice received 50 mg myristic acid/kg or 50 mg myristic-BSA/kg ip 24 hr prior to iv challenge with Naegleria (5 X 10^6/mouse) (—⊖—)(—●—) administered iv as a control. The number of animals for each experimental series was 20.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% DEAD
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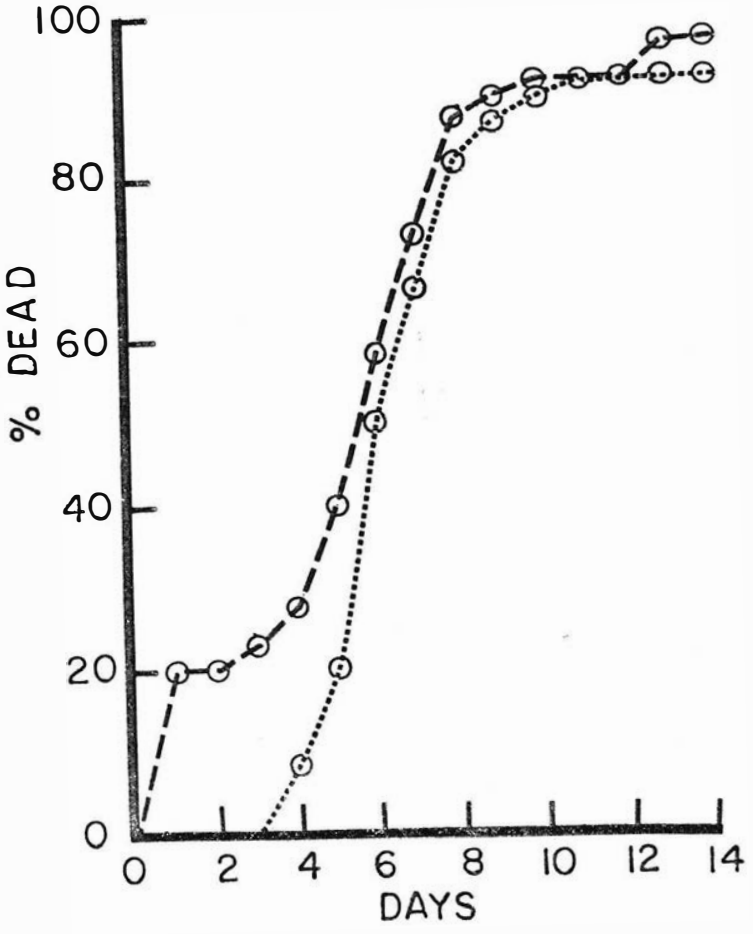
14
DAYS

Figure 11. The effect of lipid A (·····⊖·····) on the course of Naegleria infection in mice. Mice received 50 mg lipid A/kg ip 24 hr prior to iv challenge with Naegleria fowleri (Lee strain). Naegleria (5 X 10^6/mouse) (—⊖—) was administered iv as a control. The number of animals for each experimental series was 30.


100
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14
DAYS

Figure 12. Mice receiving a primary iv inoculation with 5 X 10^6 Naegleria fowleri (Lee strain)/ mouse were rechallenged after two weeks with 5 X 10^6 Naegleria/mouse iv. The number of animals for this experimental series was: 7 for iv rechallenged; 20 for control.

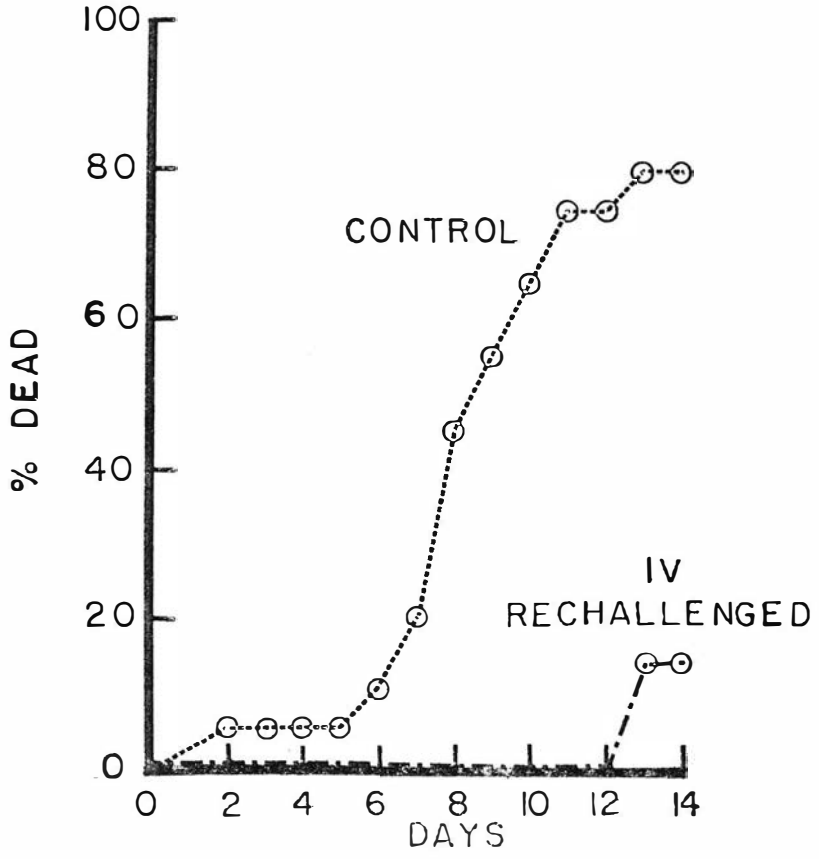
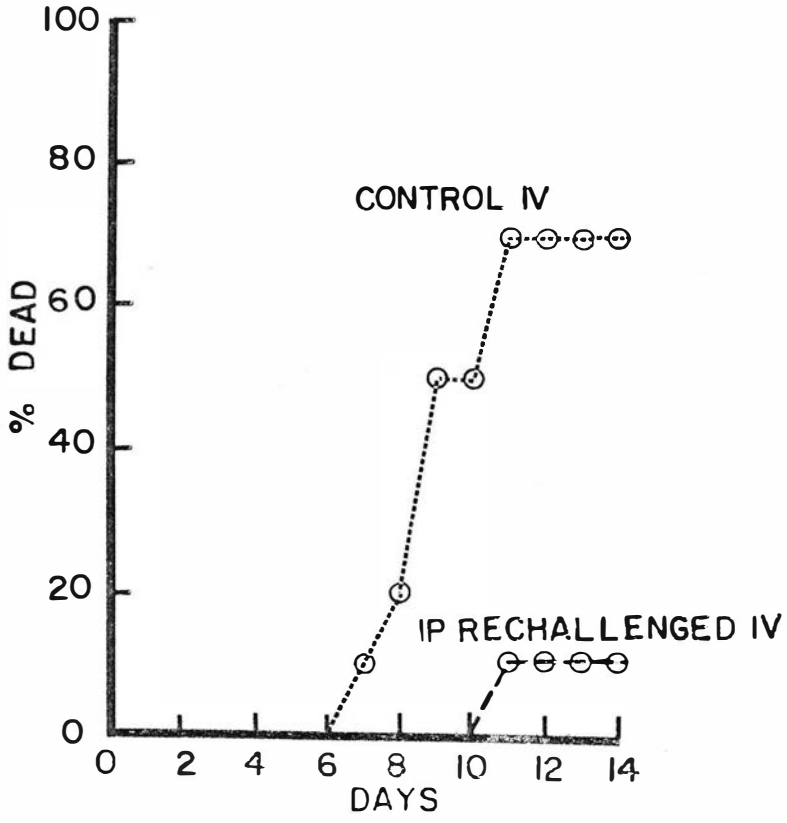

100
80
60
40
20
0
% DEAD
CONTROL
IV
RECHALLENGED
0
2
4
6
8
10
12
14
DAYS

Figure 13. Mice administered Naegleria fowleri (Lee strain) ip were rechallenged after two weeks with 5 X 10^6 Naegleria/mouse iv. The number of animals for each experimental series was 10.


100
80
60
40
20
0
% DEAD
CONTROL IV
IP RECHALLENGED IV
0
2
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DAYS

Discussion

Previous work has established that many antitumor drugs, given in combination with bacterial endotoxin, result in much greater mortality than would be expected. One of these antitumor drugs that acts synergistically with bacterial endotoxin is the plant alkaloid vincristine (Rose, 1973). A nonlethal dose of vincristine (1mg/kg) given in combination with a non-lethal dose of LPS (1mg/kg) results in the death of 20% of the treated mice (Table 3).

The literature has clearly shown that endotoxin in combination with vincristine produces one of the most potent enhancements of drug lethality known (Rose, 1973). This fact makes the vincristine treated mouse an excellent candidate for an assay system to detect small amounts of endotoxin.

It is not unexpected to find that all of the strains of Pseudomonas aeruginosa possessed endotoxic activity by the two assay systems used. It is well documented in the literature that P. aeruginosa contains endotoxin (Sadoff, 1974).

Bacteroides, being a gram-negative organism, would also be expected to show endotoxic activity in the vincristine-treated mouse as well as by eliciting protection to the lethal action of LPS. Four of the seven strains of Bacteroides showed endotoxic activity in the vincristine-treated mouse and all the strains protected mice against subsequent challenge with a lethal dose of LPS. Sonnenwirth et al. (1972) have shown that endotoxin is present in LPS extracts from strains of B. fragilis and B. melaninogenicus by the Limulus assay. LPS preparations from B. fragilis and B. melaninogenicus contained ketodeoxyoctonate

which is a common constituent of bacterial endotoxin (Sonnenwirth et al., 1972).

Since Bacteroides constitutes a large portion of the normal flora of man (Moore and Holdeman, 1974) and bacteremia due to Bacteroides has increased over the last 10 years (Sonnenwirth et al., 1972) the role of endotoxin in anaerobic gram-negative bacteremia needs to be clarified.

Listeria monocytogenes differs from the above organisms in that it is a gram-positive organism. An extract from the cell wall of Listeria has been isolated and reported to have many of the biological activities of endotoxin (Potačka et al.,1974, John et al., 1974, and Srivastava and Siddique, 1975). Endotoxic activity was found in both assays for heat-killed Listeria strains 19303, PW, D9, and H-3. No endotoxic activity was found for strains MCV, V-5, T_4b and FH-1. Cells of strain T_3b produced increased resistance to LPS but did not kill vincristine-treated mice.

MCV, 19303, PW, T_4b and T_3b strains were human isolants and pathogenic for mice whereas H-3, V-5, V-6, FH-1 and D-9 strains were isolated from vegetation and considered avirulent. Endotoxin-like activity did not seem to be contingent on whether the organism was virulent or avirulent.

The lack of endotoxic activity of some of the strains of Bacteroides and Listeria in the vincristine treated mouse may be due to the use of whole bacteria in which the endotoxin might not have been released before the vincristine had been excreted. Alternatively LPS might have been released into the growth medium in the case of Bacteroides and Listeria or washing fluids in the case of Listeria.

All three of the above organisms are easily isolated from man or the environment and are not usually pathogenic except in the compromised host. The clinician should be aware that synergies with antineoplastic agents (such as VNC) and endotoxin-like substances from opportunistic organisms could cause death in patients concurrently receiving cytotoxic drugs such as antineoplastic agents and immunosuppressants.

Previous work has established that bacterial endotoxins may enhance or inhibit the pathogenicity of infection depending on the infecting microorganism, dose and route of injection of endotoxin, and the interval between administration of toxin and initiation of infection. Effects of endotoxin on resistance have been demonstrated in infection by protozoa (Trypanosoma, Plasmodium), fungi (Candida, Rhizopus, Blastomyces, Histoplasma, Cryptococcus), viruses (influenza, ectromelia, encephalitis, and New Castle disease), gram-positive bacteria (Staphylococcus, Pneumococcus, Streptococcus), gram-negative bacteria (Klebsiella, Pseudomonas, Salmonella, Escherichia coli), and Mycobacterium (Cluff, 1971). The mechanism for this alteration of infection is not known. Endotoxin affects the cellular and humoral mechanisms that control resistance. Also the action of endotoxin may be altered by an animal's nutritional state, metabolic state, age, sex, and other constitutional characteristics.

Naegleria fowleri infection was used to determine if endotoxin and endotoxin-like material could alter the course of infection. The mouse was chosen as the experimental animal because the existence of primary amebic meningoencephalitis in man was suggested following the discovery of fatal meningoencephalitis in mice after intranasal inoculation of a species of Acanthameba. When primary amebic meningoencephalitis was subsequently reported in humans, the remarkable similarity

of the disease occurring in man to that produced experimentally was promptly recognized (Culbertson et al., 1958). Also, the basic features of the disease in man have all been noted in experimental infections in the mouse namely the same incubation period and portal of entry and central nervous system involvement (Martinez et al., 1973).

The intranasal route of inoculation has been the classical way of inoculating mice or most experimental animals with Naegleria since it has been thought to be the route of entry in primary amebic meningoencephalitis in man. Small numbers of Naegleria administered by the intranasal route are able to produce primary amebic meningoencephalitis in experimental animals. However, with intranasal inoculation it is extremely difficult to quantitate the number of trophozoites given.

Mice were inoculated by the subcutaneous, intraperitoneal, and intravenous routes. Clinical symptoms and death were produced in mice inoculated via the intravenous and intraperitoneal route. Only one dose (5×10^6 trophozoites/mouse) was administered subcutaneously. Since the course of infection of the Naegleria infection seems to be dose dependent via the iv and ip route it is reasonable to propose that a larger dose can produce the disease in mice via subcutaneous inoculation.

Intravenous inoculation was chosen over other routes of inoculation for subsequent experiments because it produced clinical symptoms and death in mice in a reasonable amount of time (5-7 days) and required a reasonable number of trophozoites (5×10^6 trophozoites/mouse).

Culbertson et al. (1968) reported inoculation of mice with Naegleria via the intranasal as well as the intraperitoneal, intravenous and intracerebral routes. According to Culbertson et al. if 100-10000 Naegleria were instilled intranasally, mice died in 8-10 days of "rhinen-

cephalitis". They also stated that wide hematogenous dissemination was seen after intravenous and intraperitoneal administration (Culbertson et al., 1968). No tables, figures or doses for Naegleria administered iv or ip are available.

In 1972 Culbertson et al. reported inoculating Naegleria into subcutaneous and skeletal muscle tissue of guinea pigs and specific pathogen free mice. Doses of 8×10^4 to 1×10^5 amebae were administered im or subcutaneously. It was determined that most guinea pigs died without central nervous system involvement after receiving 1×10^4 amebae. It was stated that comparable experiments were repeated in SPF mice and that mice were not as susceptible as guinea pigs to infection by Naegleria after subcutaneous injection. Again no dose of Naegleria was reported (Culbertson et al., 1972).

Carter (1970) studied the pathogenicity of the Naegleria by various routes of inoculation. Swiss Webster mice were inoculated in groups of 3 by the intranasal, intragastric, intrarectal, intravenous, intramuscular, subcutaneous, intraperitoneal, intrahepatic, intrapleural, anterior intracerebral, and posterior intracerebral routes. A dose of 2×10^4 Naegleria in proteose peptone glucose medium with a pH of 6.5 and a molarity of 0.135 M was administered each mouse. Clinical symptoms and death occurred only in mice administered Naegleria intranasally, anterior intracerebrally, and posterior intracerebrally. One out of three mice died with no clinical symptoms in both of the groups administered Naegleria intravenously and intrahepatically. No clinical or pathological evidence of disease was found when Naegleria were administered via the intrarectal, intragastric, intramuscular, intraperitoneal, subcutaneous, or intrapleural routes (Carter, 1970).

Wong (1975) inoculated Naegleria via the intranasal, intrathecal, and intravenous routes into monkeys. Wong administered 1 X 10^4 to 4 X 10^5 Naegleria/monkey intranasally, 2.8 X 10^4 to 3 X 10^5 Naegleria/monkey intravenously and 2.5 X 10^4 to 1 X 10^6 Naegleria/monkey intrathecally. No clinical symptoms or death resulted in monkeys inoculated intranasally or intravenously using the doses of Naegleria described above. Clinical symptoms occurred in all monkeys and death in some monkeys inoculated intrathecally (Wong, 1975).

The literature on route of inoculation of Naegleria is not convincing because too few animals composed the experimental groups, the numbers of trophozoites administered were not reported or too small a dose of amebae was administered, and the ages of the Naegleria cultures were not stated.

Mice pretreated with bacterial LPS 24 hr prior to iv challenge with Naegleria were afforded protection early in the course of infection. This early protection from naeglerial infection may have been due to a response of the host to LPS which caused products to be formed or cells to be mobilized in the host that were deleterious to the Naegleria. Since the ability of Naegleria to establish a progressive disease is dose dependent, survival times of the mice would be increased if the numbers of Naegleria were decreased by the host response.

Mice pretreated with LPS were definitely protected from the acute deaths that were occasionally observed in mice inoculated iv with Naegleria alone. These acute deaths may have been caused by the toxin that is reportedly produced by Naegleria (Chang, 1971).

None of the other endotoxin-like substances tested afforded protection from Naegleria infection in mice except possibly lipid A. According to Lüderitz et al. (1973), lipid A is the active toxic component of LPS.

They reported that lipid A complexed with certain carriers was less active than its parent LPS but did show endotoxic activity in the following assays: mouse lethality, pyrogenicity, bone marrow necrosis, Limulus gelation and complement inactivation (Lüderitz et al., 1973). Thus it was disappointing that the lipid A complexes showed no alteration of the course of the naeglerial infection. Lipid A alone prolonged survival time of mice early in the infection. By day 6 post injection there was no significant protection. Significant protection was afforded mice from acute deaths observed in mice administered Naegleria alone.

Mice that survived a primary dose of Naegleria iv or ip were significantly protected from a rechallenge with Naegleria iv. This effect can probably be attributed to increased immunity of the host to naeglerial infection. The initial challenge of Naegleria acted as a vaccine.

The possibility that a latent infection might become manifest and produce disease in a compromised host prompted the experiment using cyclophosphamide on survivors. The experiment did not have any controls to show that the dose of cyclophosphamide used immunosuppressed the animals.

The study presented here introduces many questions that need to be pursued. Some stem from subjective observations made during the research. One observation is that Naegleria seem to become more virulent after frequent transfer of the cultures in culture media. Naegleria can be transferred with varying time intervals between transfers and dose response in mice results calculated. Along this same vein, nonpathogenic Naegleria might be rapidly transferred in culture media and administered to mice to see whether death could be produced.

The question of the cause of acute deaths in mice administered Naegleria iv and ip needs to be resolved. Is it a toxin? One way to

determine whether Naegleria must be present for acute deaths to occur would be to inoculate mice with the supernatant fluid from the Naegleria suspension that has been prepared in the normal manner for iv inoculation.

Since it has been shown previously that time of administration of LPS influences the degree of protection (MacGregor et al., 1969), many doses of LPS should be used to pretreat mice and varying time of administration of LPS should be tried.

Tolerance or increased resistance to the deleterious effects of bacterial endotoxins ensues after exposure of experimental animals to a single or multiple injections of such toxins (Moreau and Skarnes, 1973). MacGregor et al. (1969) failed to show protection from malaria infection in LPS tolerant mice. Singer et al. (1964) have shown that animals tolerant to LPS were protected from trypanosome infection. Because of varying reports, it would be interesting to see if the course of naeglerial infection could be altered in the LPS tolerant mouse.

The question of the course of infection of Naegleria in the immunosuppressed mouse is still unanswered. Experiments should be done to determine a dose of drug that would immunosuppress the animal. Then the dose could be used on survivors to see if one could produce the disease in a compromised host. The immunosuppressive drug could also be used to see if a compromised host was more susceptible to the infection than a "normal" animal.

A survey of drugs would be advantageous to determine if Naegleria infection could be inhibited or cured. One should initiate this study with drugs that are able to cross the blood brain barrier. Time of administration of drugs would probably also be important.

Protection of animals from a secondary challenge using doses of live Naegleria iv and ip has already been shown. Protection of mice from

naeglerial infection might be afforded by using frozen or heat killed cells as a vaccine. One might also administer live trophozoites in multiple low doses and challenge with a large dose of Naegleria to see if animals are protected. Doses of nonpathogenic Naegleria might also be used as a vaccine.

Many interesting experiments are left to be done with naeglerial infection in mice. These experiments may answer many of the questions that make this organism such a mystery. Can a common soil ameba Naegleria become a pathogen? If so, is it the environment or the host or both?

SUMMARY

In this study the distribution and effects of bacterial endotoxin were examined. Adult male BALB/c and adult male DUB/ICR mice were used in all experiments. Endotoxic activity of a number of strains from three genera of bacteria, Pseudomonas, Bacteroides, and Listeria, was detected using vincristine-treated mice as sensitized indicators for endotoxin and resistance to purified endotoxin induced by pretreatment with selected bacteria. Also, the effect of bacterial endotoxin and endotoxin-like substances on the course of primary amebic meningoencephalitis in mice, caused by Naegleria fowleri, was assessed.

Cells from 10 mucoid strains of Pseudomonas aeruginosa, isolated from patients with cystic fibrosis, were harvested, suspended in saline and killed by heating in a boiling water bath for 10 min. Mice were administered a cell-suspension having an absorbance at 420 nm of about 10(0.01 ml/gram of mouse) simultaneously with 1 mg vincristine /kg. All 10 strains of Pseudomonas possessed endotoxic activity. Moreover treatment of mice with heat-killed Pseudomonas rendered the animals resistant to a dose of lipopolysaccharide (LPS) that killed untreated mice. Cells from 7 strains of Bacteroides were harvested, suspended in saline and frozen. Mice were administered a cell-suspension having an absorbance at 420 nm of about 40(0.01 ml/gram mouse). Four of the Bacteroides strains possessed endotoxic activity by this assay. All 7 strains were able to evoke resistance to a lethal dose of LPS. Cells from 10 strains of Listeria monocytogenes were examined; about half of these possessed endotoxin-like activity by both assay systems used.

The course of the disease after infection of mice with Naegleria fowleri was dose dependent, whether challenged via the intraperitoneal (ip) or intravenous (iv) route. After iv administration of 1 X 10^7 trophozoites/mouse 100% of the mice were dead by day 10 as compared to 70% mortality on day 10 for a dose of 5 X 10^6 Naegleria/mouse and 40% mortality on day 10 for a dose of 2.5 X 10^6 trophozoites/mouse. Mice administered 5 X 10^6 and 1 X 10^7 trophozoites by the ip route had 10% and no mortality by day 18 respectively. However, all the mice receiving 4.85 X 10^7 Naegleria ip were dead by day 8. Mice were pretreated with LPS (1mg/kg) and endotoxin-like materials (50mg/kg) 24 prior to iv challenge with Naegleria fowleri (5 X 10^6 trophozoites/mouse). Mice pretreated with Escherichia coli 026:B6 LPS before challenge, displayed an increase in resistance during the early portion of the naeglerial disease. Mice pretreated with endotoxin-like materials such as lipid A complexed to bovine serum albumin (BSA), lipid A complexed to concavalin A (Con A), bovine serum albumin, concanavalin A, beta hydroxymyristic acid, beta hydroxymyristic-Con A, myristic acid, myristic-BSA, N,N dimethylmyristic-BSA and N,N dimethylmyristic acid were afforded no protection from the naeglerial infection.

Mice that survived a primary iv or ip challenge with Naegleria fowleri were rechallenged iv with 5 X 10^6 Naegleria/mouse. These mice were significantly protected from the second challenge with Naegleria fowleri. The initial challenge with Naegleria acted as a vaccine.

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