Viral Neuroinvasion and Encephalitis Induced by Lipopolysaccharide and its Mediators

By Shlomo Lustig, Haim D. Danenberg, Yaron Kafri, David Kobiler, and David Ben-Nathan

From the Department of Virology, Israel Institute for Biological Research, Ness-Ziona 70450, Israel

Summary

The present study was designed to test the effect of bacterial endotoxin on penetration of viruses into the central nervous system (CNS). As a model we used two neurovirulent viruses that lack neuroinvasive capacity: West Nile virus-25 (WN-25) and neuroadapted Sindbis virus (SVN). Administration of lipopolysaccharide (LPS, 100 μg/mouse) to CD-1 mice, followed by WN-25 inoculation resulted in 83% encephalitis and death, compared with <5% in controls. The results in SVN-inoculated CD-1 mice were quite similar. LPS-treated mice suffered 62% mortality compared with 6% in the nontreated group. No changes in viral neuroinvasiveness were demonstrated in viruses isolated from brains of encephalitic mice, suggesting that neuroinvasion is not due to a selection process for an invasive variant, but to direct penetration of the viruses through the blood-brain barrier (BBB). LPS did not induce WN-25 encephalitis in LPS-insensitive C3H/HeJ mice, compared with 100% neuroinvasion in C3H/HeB mice. Induction of neuroinvasion could be transferred to C3H/HeJ mice by transfusion with serum obtained from LPS-treated, LPS-responsive mice. Passive immunization of CD-1 mice with anti-mTNF antibodies before LPS administration did not prevent LPS-induced WN-25 encephalitis. Furthermore, neutralization of tumor necrosis factor activity in the serum of LPS-treated mice did not abolish its activity, and transfusion-associated encephalitis was observed after the administration of the neutralized serum with WN-25.

We suggest that LPS can contribute to virus penetration from the blood into the CNS, a process which turns a mild viral infection into a severe lethal encephalitis. This effect is mediated by soluble factors, and is probably achieved by injury to cerebral microvascular endothelium and modulation of BBB permeability.

Neuroinvasiveness (ability to penetrate the central nervous system [CNS][1]) and neurovirulence (ability to establish a lethal infection within the CNS) are the crucial elements that determine the capacity of a virus to cause encephalitis (1, 2). The major routes for virus spread into the brain are through nerves or via the bloodstream. Arboviruses, which are the most common known cause of encephalitis worldwide (3, 4), usually spread hematogenously. The blood-brain barrier (BBB), ultrastructurally localized at the cerebral microvascular endothelium, presents a major obstacle to neuroinvasion from the blood (1, 2, 5).

The mode and specificity of viral entry through the BBB into the CNS remains obscure. Factors other than inherent genetic viral traits (1, 6–8) can influence neuroinvasion and allow entrance of noninvasive viruses into the CNS. Among such factors are modulation of BBB permeability by hyperosomotic agents, hypercarbia, hypothermia, and mechanical breach (9–12).

Modulation of BBB permeability was also found in experimental bacterial meningitis. Functional and ultrastructural changes of the BBB were revealed in animals challenged with live bacteria or with bacterial LPS (13, 14).

The present experiments were designed to test whether systemic administration of LPS will lead to viral neuroinvasion in a mouse model. To examine our hypothesis, that increased permeability of the BBB facilitates viral neuroinvasion, we used two neurovirulent but noninvasive viruses, a flavivirus, West Nile virus-25 (WN-25) (15, 16) and an alphavirus, neuroadapted Sindbis virus (SVN) (12, 17).

The results presented herein clearly demonstrate that LPS is very effective in the induction of viral encephalitis by these otherwise noninvasive viruses.

[1] *Abbreviations used in this paper:* BBB, blood-brain barrier; CNS, central nervous system; DS, donors' serum; SVN, neuroadapted Sindbis virus; WN-25, West Nile virus-25.

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Materials and Methods

Mice. Female CD-1 mice were obtained from Charles River (London, UK), and C3H/HeB and C3H/HeJ mice were obtained from The Jackson Laboratories (Bar Harbor, ME). The mice used were aged 4–5 wk-old. In all experiments, mice of the same age and batch were used.

Viruses. The isolation of WN-25 has been described previously (15, 16). Briefly, wild-type WNV was passed in *Aedes aegypti* cultures. At passage 25, the virus was plaque purified and kept in stock. Mice were inoculated intravenously with 0.2 ml containing 2×10^5 PFU. Virus dilutions were performed using PBS containing 2% FCS.

The isolation of SVN has been described previously (12, 17). Briefly, a nonvirulent strain of Sindbis virus was passed by consecutive intracerebral injections in suckling mice. A new passage variant was isolated at the 15th passage and was lethal to weanling mice when injected intracerebrally, but not when injected intraperitoneally. Mice were inoculated intravenously with 0.2 ml containing 1.5×10^5 PFU. Virus dilutions were performed as for WN-25.

LPS Challenge. Mice were injected intraperitoneally with LPS (055/B5; Difco Laboratories, Inc., Detroit, MI) diluted in pyrogen-free saline followed by virus injection into the tail vein. Mice were observed for mortality for 14 d. To determine changes in viral neuroinvasive capacities, dying mice were killed and virus was isolated. The technique used for isolation of virus from brain tissue is described elsewhere (16). The isolated virus was tested for neuroinvasion by peripheral inoculation of naive weanling CD-1 mice.

Transfusion with Sera from LPS-treated Mice. Blood was drawn from LPS-treated (100 μg/mouse) mice into serum separator tubes (Becton Dickinson & Co., Mountain View, CA) and the serum was pooled (donors' serum [DS]). DS was used fresh, after heat inactivation (56°C, 30 min) or stored at −70°C until further processing. WN-25 was diluted in serum to 10^6 PFU/ml, and 0.2 ml were injected intravenously to naive mice.

Blocking of TNF Effects. To block TNF-mediated phenomena that are induced by LPS, we passively immunized mice against murine TNFα. Mice were injected intraperitoneally with 5×10^4 neutralizing units of antimurine TNF antibodies (10^6 U/ml, Genzyme Corp., Boston, MA) 4 h before challenge.

To block the effect of TNF in transfusion-associated encephalitis, DS was mixed 10:1 with rabbit anti-TNF antibodies, and incubated for 60 min at 37°C. Virus dilutions were then done, and the virus-containing solution was injected as described above.

TNF bioactivity in the serum was assayed using A-9 cells. Briefly, 3×10^4 cells (American Type Culture Collection, Rockville, MD) were seeded in wells of microtiter plates and allowed to adhere overnight. Wells were treated with serial dilutions of the sera followed by addition of cycloheximide (25 μg/ml). After 8–10 h incubation, the wells were washed and the number of surviving cells was determined by uptake of neutral red. Standard curves of human recombinant TNF were run on each plate. Results are weight equivalents of human TNF activity.

Data Analysis. Differences in mortality were assessed using the two-tailed Fisher's exact test.

Results

LPS-induced Encephalitis. The experiments summarized in Table 1 were done to determine whether LPS affects WN-25 neuroinvasion. CD-1 mice were injected intraperitoneally with various nonlethal doses of LPS, 2 h before WN-25 inoculation. Death was recorded in 50% of the mice treated with a 10-μg dose of LPS and in 80% of those treated with a 100-μg dose. Death occurred on days 7–9 after inoculation. The mean time of death was similar in groups injected with different LPS doses.

Table 1. *Dose Response of Mortality from WN-25 Encephalitis after LPS Treatment*

LPS dose (μg/mouse)	Dead/total	Percent
0	1/16	6
1	1/16	6
5	2/16	12
10	10/20	50*
25	9/16	56*
50	14/16	87.5*‡
75	13/16	81*‡
100	18/20	90*‡
200	15/16	94*‡

CD-1 mice were injected intraperitoneally with LPS (diluted in pyrogen-free saline to indicated doses), followed 2-h-later by intravenous inoculation with WN-25 virus (2×10^5 PFU/0.2 ml). Mortality was observed on days 7–9. No additional mortality was observed until 21 d after inoculation.
* $P \leq 0.01$ vs. no LPS.
‡ $P \leq 0.01$ vs. 10 μg LPS.

Choosing 100 μg as an effective neuroinvasion-inducing dose ($10LD_{50}$), we investigated the significance of different schedules of virus inoculation on mortality. Inoculation of virus immediately after LPS or 24 h later resulted in a similar mortality rate. Inoculation with WN-25 48 h after LPS injection caused death in five of the eight animals injected (62.5%). The mortality of mice inoculated at 72 h after LPS was not different from that of mice not treated with LPS.

The viral inoculum used in those experiments was chosen after previous studies that examined WN-25 neuroinvasion (16). When the inoculum was reduced, it was found that

Table 2. *Relation between WN-25 Dose and LPS-induced Encephalitis*

Infecting dose (PFU/mouse)	Dead/total	Percent
2×10^6	6/7	86
2×10^5	7/8	88
2×10^4	4/7	57
2×10^3	1/6	17
2×10^2	0/6	0

CD-1 mice were inoculated intravenously with WN-25 at indicated doses 2 h after administration of LPS (100 μg/mouse). Mortality was recorded on days 7–9. No mortality was observed thereafter.

LPS neuroinvasion-inducing activity is dependent upon the infecting dose (table 2). LPS was still effective when the WN-25 dose was reduced to 2 × 10^4 PFU per mouse, but not at lower WN-25 doses. Thus, an inoculum of 2 × 10^5 was used in successive studies.

To rule out the possibility that LPS-induced encephalitis is unique for WN-25 virus, the experiments were repeated with SVN. Again, mortality was observed on days 7–9 in the LPS-treated mice. Death rate was 62.5% with only 6% mortality in the SVN-infected, nontreated mice (Fig. 1).

To prove the specificity of the LPS-induced neuroinvasion phenomenon, we studied this experimental model in LPS-insensitive mice. Nontreated C3H/HeJ mice reacted the same as CD-1 and C3H/HeB mice to intravenous and intracerebral WN-25 challenge. When the three strains were treated with LPS followed by WN-25 inoculation, the mortality rate in the C3H/HeJ mice was 12.5% compared with 100% in C3H/HeB mice and 83% in CD-1 mice (Table 3).

Viral Neuroinvasive Capacity. Neuroinvasion could be the consequence of the LPS-induced selection process that results in selection of a neuroinvasive strain. To eliminate this possibility, we examined the virus isolated from brains of six mice with LPS-induced encephalitis. Naive CD-1 mice were inoculated intraperitoneally or intracerebrally with that virus. No mortality was observed in all mice injected intraperitoneally with 2 × 10^5 PFU, whereas 100% mortality was observed in mice injected intracerebrally with 10 PFU. The ratio between titers (PFU/ml, intracerebral LD_{50}, intraperitoneal LD_{50}) was similar to that of the original WN-25 (16), indicating that no changes occurred in the neuroinvasive properties of the virus. The same findings were demonstrated with the virus recovered from the brains of SVN-encephalitic mice.

Transfusion Studies. These experiments were planned to determine whether LPS-induced neuroinvasion is mediated by soluble factors. The DS obtained from CD-1 mice (donors) after LPS challenge (100 μg/mouse) was mixed with virus

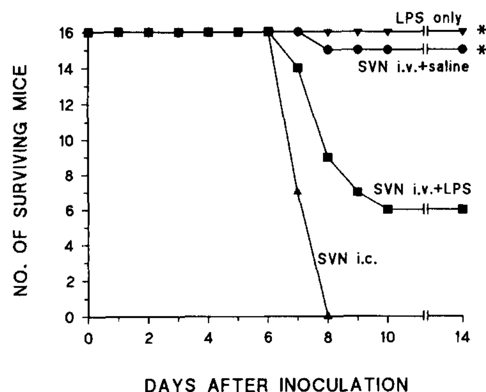

Figure 1. Induction of SVN encephalitis and mortality by LPS. CD-1 mice were injected intraperitoneally with LPS (100 μg/mouse) or pyrogen-free saline, followed 2 h later with SVN (1.5 × 10^5 PFU/0.2 ml i.v.). Mice inoculated intracerebrally were given 10 PFU in 0.03 ml PBS. No additional mortality was observed until 21 d after inoculation. $P \leq 0.01$ when compared with the SVN/saline or the LPS only groups.

Table 3. *Mortality from WN-25 Encephalitis in LPS-treated CD-1, C3H/HeB, and C3H/HeJ Mice*

Treatment	Mouse strain	Dead/total	Percent
LPS 100 μg	CD-1	0/20	0
	C3H/HeB	0/10	0
	C3H/HeJ	0/8	0
WN-25 intravenous	CD-1	2/45	4
	C3H/HeB	0/8	0
	C3H/HeJ	0/8	0
WN-25 intracerebral	CD-1	20/20	100
	C3H/HeB	10/10	100
	C3H/HeJ	8/8	100
LPS + WN-25	CD-1	88/106	83*
(intravenous)	C3H/HeB	20/20	100
	C3H/HeJ	5/40	12.5‡

LPS (100 μg/mouse) or pyrogen-free saline was injected intraperitoneally 2 h before inoculation with WN-25 (2 × 10^5 PFU in 0.2 ml of PBS with 2% FCS, intravenous) To check viral neurovirulence mice were injected intracerebrally with 10 PFU in 0.03 ml PBS. Mice were observed for mortality for 14 d.
* $P \leq 0.01$ compared with control.
‡ $P \leq 0.01$ compared with C3H/HeB mice.

and injected to naive mice. LPS levels were assayed in the DS using the limulus lysate test. In three samples that were assayed at 2 h post-LPS, levels were below 100 ng/ml. Thus, the LPS dose injected in DS could not exceed 20 ng/mouse. To abolish this residual LPS activity, which is far below the minimal effective dose, C3H/HeJ mice were selected to be used as recipients. Injection of DS (obtained at 90–120 min after LPS) mixed with WN-25 induced encephalitis in 75% of the C3H/HeJ mice and in 78.8% of CD-1 mice. No effect was observed with sera obtained from LPS-injected C3H/HeJ mice. When DS obtained at 6 h after challenge was used, the effect was diminished to 14% encephalitis rate. DS obtained at 24 h after challenge was not at all effective. Heat inactivation of DS almost completely abolished encephalitis induction, whereas storage of the serum at −70°C did not affect its activity (Table 4).

Neutralization of TNF Effects. TNF is a major mediator of LPS pathophysiologic sequela which is found in high levels 1–2 h after LPS administration. We investigated whether blocking the LPS-induced TNF response would effect the induction of WN-25 encephalitis. TNF levels in the serum were assayed in control CD-1 mice 90 min after LPS (100 μg/mouse) and were found to be 9.3 ± 2.8 ng/ml (n = 8, mean ± SEM). CD-1 mice were passively immunized against TNF using rabbit polyclonal antimurine TNFα antibodies. No TNF activity was detected in the sera of immunized mice at 90 min after LPS. Passive immunization

Table 4. *The Effect of Post-LPS Serum on the Induction of WN-25 Encephalitis*

Challenge	Recipient mice	Dead/total	Percent
DS	C3H/HeJ	6/8	75
DS	CD-1	41/52	79
DS	C3H/HeB	7/8	87.5
DS (from C3H/HeJ)	CD-1	1/9	11
Control sera	CD-1	0/8	0
	C3H/HeB	0/8	0
	C3H/HeJ	0/8	0
DS (+56°C*)	CD-1	0/8	0
DS (−70°C‡)	CD-1	7/8	87.5
DS (6 h§)	CD-1	2/14	14
DS (24 h‖)	CD-1	0/14	0

DS were obtained from CD-1 mice (unless indicated otherwise) 90–120 min (unless indicated otherwise) after LPS (100 μg/mouse). WN-25 virus was diluted twofold in DS and 0.2 ml were injected intravenously. Control mice were injected with WN-25 diluted in serum of naive CD-1 mice.
* DS incubated in 56°C for 1 h before dilution with virus.
‡ DS stored in −70°C until use.
§ DS drawn 6 h after LPS challenge.
‖ DS drawn 24 h after LPS challenge.

resulted in a mild delay in the time of death (statistically nonsignificant), but no difference was observed in LPS-induced WN-25 encephalitis and death rates.

To assess TNF involvement in transfusion-associated encephalitis, DS was incubated with anti-TNF antibodies. No TNF activity was detected by bioassay in incubated DS, but it still effectively induced WN-25 neuroinvasion and encephalitis in naive CD-1 and C3H/HeJ mice.

Discussion

The present experiments demonstrate that systemic administration of LPS to mice can induce viral neuroinvasion and encephalitis.

As a model we used two viruses that lack neuroinvasive capacities, but kill mice when injected intracerebrally. Neuroinvasion is easily evaluated in these models, as brain penetration by <10 PFU of either one of these viruses results in the development of severe lethal encephalitis 7–9 d after inoculation (11).

The ratio between asymptomatic infection and clinical disease varies for the different neurotropic viruses. However, asymptomatic or mild infections are extremely common (3, 4). The factors responsible for the progression of asymptomatic or mild infections to life-threatening encephalitis are not defined. Our data suggest that infection with gram-negative bacteria may play a role in the determination of the clinical course of infection with a neurotropic virus. Such infection, either recent (48 h earlier in our study) or concurrent, can turn a mild infection into a severe encephalitis. Infections with gram-negative bacteria are extremely prevalent, especially in the young and in the elderly—the same age groups that are most commonly affected by viral encephalitis (18).

WN-25 virus can cause encephalitis in mice subjected to isolation or repeated cold stress (16). The virus that was isolated from brains of mice with such stress-induced encephalitis was different to the virus injected, by virtue of a neuroinvasive capacity, similar to that of wild-type WNV. Neuroinvasion was then attributed to immunosuppression with increased replication, allowing back-mutation and selection of an invasive strain. That was not the case in the present study. The virus that was isolated from brains of encephalitic mice was similar to the virus with which they were inoculated, and it did not demonstrate any invasive traits. Thus, it seems that LPS-induced neuroinvasion is due to altered host mechanisms, rather than the result of a change in viral traits. Heinz et al. (19) studied the molecular basis of attenuation in flaviviruses, and suggested that the loss of the neuroinvasive capacity is due to a defect in the domain B of the viral protein E. The ability of this protein to bind to a specific receptor was presumed to establish the neuroinvasive capacity. We speculate that in our model, LPS made it possible to bypass the binding to receptor process, which is otherwise mandatory. Thus, considering the ability of LPS to increase the permeability of the BBB (13, 14), we suggest that neuroinvasion is achieved by injury to the BBB and penetration of viruses through the breach.

C3H/HeJ mice are resistent to LPS toxicity because of a defective immunologic response to LPS, which includes a defect in TNF production (20, 21). LPS did not induce neuroinvasion in these mice in contrast with the high rates of neuroinvasion observed in CD-1 and C3H/HeB mice. The absence of encéphalitis in LPS-treated C3H/HeJ mice indicates once again that the host response was the major determinant of neuroinvasion in our model. Encephalitis was observed after transfusion of C3H/HeJ mice with serum obtained from LPS-sensitive mice challenged with LPS. This transfusion-associated neuroinvasion points to a soluble mediator or mediators, probably of lymphoid origin, that can independently induce neuroinvasion. The lack of activity in heat-inactivated serum suggests proteins as these mediators. The lack of activity of DS obtained 6 h after LPS indicates a short mediators response. However, LPS is effective in inducing neuroinvasion even if the virus is inoculated as late as 48 h. Thus, it seems that a short mediators response after LPS enable neuroinvasion for a period over 48 h.

TNF is a major mediator of LPS pathophysiological sequela, which is found in high levels in the serum at 1–2 h after LPS challenge and disappears at 5 h (22). Existing evidence implies that it is capable of increasing the permeability of microvascular endothelium throughout the body (23, 24), including that of the BBB (25). Based on these studies, we hypothesized that TNF is the mediator of LPS-induced neuroinvasion. However, this theory was negated since we showed that viral neuroinvasion was not prevented in LPS-challenged

mice that were passively immunized against TNF. No conclusions can yet be drawn on the contribution of TNF response to neuroinvasion. Our data indicate that it may not be an essential direct mediator of LPS-induced and transfusion-associated neuroinvasion.

The pathophysiological sequela of LPS administration involves activation of the cells of the mononuclear phagocyte system with increased production and release of several mediators including IL-1. IL-6, interferons, histamine, and TNF (21, 26, 27). Further experiments would evaluate the effects of these mediators on neuroinvasion and whether they are involved in LPS-induced neuroinvasion.

When WN-25 and the wild-type WNV were compared (28), it was found that apart from different plaque sizes in Vero culture, the only difference in biological activity was in their ability to replicate in macrophage cultures. Whereas adsorption to macrophages was similar for both viruses, replication was some 100-fold-higher for the wild-type. Adsorption of neurotropic viruses to macrophages, with and without amplification, has been described with several other viruses (2, 9, 29, 30). Recently, it was shown that the cells of mice infected with dengue virus release a cytokine that induces a breakdown of the BBB (31).

Based on these findings, we suggest that the difference between invasive and nonneuroinvasive viruses could be found in the virus' ability to stimulate the host mechanism, enabling its penetration through the BBB. Furthermore, we may speculate that macrophages and their products have a role in the pathogenesis of WNV encephalitis and in the determination of the neuroinvasive capacity. Additional studies are required to investigate the production of cytokines during infections by WNV and other neurotropic viruses, and to determine the involvement of cytokines in the pathogenesis of viral encephalitis.

Neuroinvasion of neurotropic viruses was considered to be effected by inherent viral traits (1). The present study demonstrates that LPS is capable of turning a mild viral infection into lethal encephalitis by inducing viral invasion to the CNS. This effect is mediated by endogenous mediators and is probably achieved by breaching of the BBB, which enables viral penetration to the CNS. These findings add valuable information to the understanding of the pathophysiology of viral encephalitis and may have therapeutic implications for these hazardous and lethal infections.

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Address correspondence to Dr. Shlomo Lustig, Department of Virology, Israel Institute for Biological Research, P.O. Box 19, Ness-Ziona 70450, Israel. Haim D. Danenberg is currently at Division of Internal Medicine, Hadassah Hospital, Ein-Karem, Jerusalem.

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