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Protective effects of melatonin in mice infected with encephalitis viruses

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Summary. We examined the effect of the pineal neurohormone melatonin (MLT) on protection from viral encephalitis. The antiviral activity of MLT was evaluated in normal mice inoculated with Semliki Forest virus (SFV) and in stressed mice injected with the attenuated non-invasive West Nile virus (WN-25). Administration of MLT (s.c.) daily from 3 days before through 10 days after virus inoculation reduced viremia and significantly postponed the onset of disease and death by 7 to 10 days. Moreover, MLT injection reduced mortality of SFV (10 PFU) inoculated mice from 100% to 44%. In mice inoculated with high dose of SFV (100 PFU), MLT postponed death and reduced mortality by 20%. In all of the surviving mice anti-SFV antibodies were detected 22 days after virus inoculation. Infection of mice stressed by either isolation or dexamethasone injection with WN-25 induced mortality of 75% and 50% respectively, which was reduced by MLT administration to 31% and 25%, respectively. The efficiency of MLT in protecting from lethal viral infections warrants further investigations on its mechanisms of action.

Introduction

Over the past several years, it has been recognized that melatonin (MLT) can augment the immune response and correct immunodeficiency states which may follow acute stress, viral diseases, aging, or drug treatment [15]. In humans, plasma levels of MLT are high during infancy and decline in the elderly [32]. A growing body of evidence, both experimental and epidemiological, suggests an inverse relationship between low levels of MLT in plasma and pathological situations, such as psychosomatic diseases, psychiatric and neurological disorders, and cancer [16, 27]. A similar relationship has been demonstrated between these disease and immunosuppression [1, 30]. The immunostimulating properties of MLT seem to depend on activated CD4+ T-cells, which upon MLT stimulation show an enhanced synthesis and/or release of opioid peptides, interleukin-2, and interferon-γ [17–22].

Viral infections cause an increase in glucocorticoids [3, 7, 29] and involution of thymus and spleen [4, 5] as well as a generalized immunosuppression [25, 31]. A question which arises from the above data is whether MLT may act as an antiviral agent. To clarify this question we studied the protective effect of MLT in mice infected with Semliki Forest virus (SFV) and attenuated West Nile virus. SFV has been shown previously [6] to be a suitable virus to evaluate the effect of various compounds as antiviral agents. SFV is generally considered to be of low pathogenicity for man [33]. In mice the course of disease is both predictable and more severe. After intraperitoneal inoculation, SFV replicates in skeletal muscle and fibroblasts, and causes viremia and infection of the CNS [12, 23]. Virulent strains cause a fatal encephalitis, with high titers of the virus in the brain in mice of all ages [8, 9].

Here, we report on the protective effects of MLT in mice inoculated with SFV and attenuated West Nile virus.

Materials and methods

Viruses

SFV strain B261 was obtained from the American Type Culture Collection, Bethesda, MD. Passage history: 14th passage in mouse, two passages in BHK cells. The virus stock used for experiments was prepared in BHK cells and contained 1.5×10^8 plaque-forming units per/ml (PFU/ml). The intraperitoneal titer (LD50) was 9.3×10^7/ml (mouse i.p. LD50/ml). Each mouse was inoculated i.p. with 0.5 ml of SFV containing 10 or 100 PFU/mouse. Groups of 8 to 12 mice were used and the results were evaluated according to the method of Reed and Muench [26]. The WN-25 attenuated variant of West Nile virus was isolated and cultured as described [2, 14]. Each mouse was inoculated i.p. with 0.2 ml containing 2×10^5 PFU.

Bioactive compounds

Melatonin was (a gift from Helsin Chemicals SA, Breganzona Switzerland) diluted in PBS and injected subcutaneously (s.c.) daily (500 μg/kg), starting 3 days before virus inoculation until 10 days after. Dexamethasone (Sigma, St. Louis, MO) was diluted in saline and injected intramuscularly (i.m.) 2.5 mg/kg, on days 0, 1, 2, and 3 after virus inoculation.

Mice

Charles River outbred ICR female mice (CD1) were obtained at the age of 21 days and kept in our vivarium until the age of 6 or 11 months old.

Stress

Isolation stress

Mice were housed in individual cages immediately after inoculation until the end of the experiment. Control mice were housed six per cage [2].

Isolation of virus from the blood and brain of infected mice

MLT and control mice were bled at various time points from the tail vein into serum-separator test tubes (Beckton Dickinson). Virus content in the serum was plaque-

assayed in Vero cells. Brains were dissected from moribund (taken on days 8–10 after inoculation) mice and then rinsed in cold PBS and sonicated. The virus suspension was centrifuged at 3000 rpm for 10 min. The supernatant was aliquoted into plastic tubes and stored at −70 °C until further processing. Virus levels in the brain were measured by titration of virus in Vero cells.

Titration of virus in tissue culture

For demonstration of SFV plaques in Vero cells the original plaque technique of Dulbecco and Vogt [11] was used. Serial dilutions of virus were added to Vero cell monolayers in Petri dishes which were incubated at 37 °C for 1 h to permit viral adsorption. The monolayer was overlaid with MEM × 2 and Tragacanth containing 2% FCS and 2.4% $NaHCO_3$. The cultures were incubated (37 °C, 5% CO_2) for 48 h. Plaques were counted after staining the monolayers with neutral red (0.05%).

Results

The protective effects of melatonin in mice infected with SFV

These experiments were done to determine the protective effects of melatonin in mice (6–7 or 10–11 months old) inoculated with SFV. As shown in Table 1, the mortality rate of mice treated with melatonin and inoculated with SFV (10 PFU/mouse) was 61% in 6–7 month old mice and 44% in 10–11 month old mice, as compared to 94% and 100% respectively in control infected mice. High virus levels were detected on days 8 to 12 after infection, in the brains of all the moribund mice (7.7 to 8.2 log 10 PFU/brain). MLT administration not only reduced death rate, but also significantly delayed the onset of disease and mortality.

As shown in Tables 1 and 2, MLT treatment extended the time to death in the treated mice as compared to control non-treated mice. The results show that MLT protects mice against SFV infection. The titers of antivirus antibodies in surviving mice were very high (1:640–1:1280 by HI). Virus levels in the blood of infected mice are shown in Table 3. MLT administration decreased

Table 1. The effect of melatonin on survival of 6 or 10 month old mice inoculated with 10 PFU of SFV

Treatment group	D/T	6–7 months old		D/T	10–11 months old	
		% dead	mean days to death		% dead	mean days to death
SFV	17/18	94	9.3	18/18	100	8.4
SFV + MLT	11/18	61	14.7[a]	8/18	44[a]	14.0[a]

Melatonin: 500 μg/kg, 0.5 ml S.C. daily (2 h before darkness) from 3 days before until 10 days after virus inoculation

D/T Dead/total

[a] $P < 0.05$ as compared to control group. Antibody titer of surviving 10 to 11 months old mice (22 days after inoculation) using H.I. assay was 1097 ± 118 ($n = 8$)

Table 2. Protective effect of melatonin on mortality rate of mice inoculated with 10 or 100 PFU of SFV

Treatment group	D/T	10 PFU % dead	mean days to death	D/T	100 PFU % dead	mean days to death
SFV	20/20	100	8.3	10/10	100	8.6
SFV + MLT	12/20	60[a]	12.0[a]	8/10	80	11.6

Melatonin: 500 µg/kg, 0.5 ml S.C. daily (2 h before darkness) from 3 days before until 10 days after virus inoculation

Mice: CD1 female 6 months old. *D/T* Dead/total

[a] $P < 0.05$ as compared to control

Table 3. Effect of melatonin on blood virus levels of mice inoculated with SFV

Treatment group	Log 10 PFU/ml day 1	day 2
SFV	$3.9 \pm 0.2(10)$[a]	$2.8 \pm 0.2(6)$
SFV + MLT	$2.1 \pm 0.6(10)$	$1.4 \pm 0.5(6)$

Mice: 10 month old CD1 female. Number of mice tested are shown in parenthesis

[a] $P < 0.05$ as compared to MLT group

the virus levels in blood as compared to infected control mice. MLT treatment moderated the virus level in the blood on day 1 (3.9 vs. 2.1 log 10 PFU/ml) and on day 2 (2.8 vs. 1.4). In all experiments, the level of virus in the blood in MLT-treated mice was lower than in nontreated mice.

Effect of melatonin on involution of lymphoid organs of mice exposed to stress

Stress is known to induce involution of lymphoid organs such as the thymus, spleen, and lymph nodes [2, 3, 28]. This involution serves as a common marker for assessing the immunosuppressive effect of stress. The purpose of these experiments was to determine if MLT can prevent this stress-induced involution.

Table 4 presents the thymus, spleen, and body weight of mice subjected to isolation stress or following dexamethasone injection and treated with MLT. The weight of the spleen and thymus was reduced in isolation stress or following dexamethasone injection ($P < 0.05$). This effect was antagonized by MLT treatment in isolation stress or dexamethasone groups ($P < 0.01$). MLT pretreatment was found to be effective, in both stress paradigms, in preventing the involution of lymphoid organs.

Table 4. Effect of melatonin on involution of the lymphoid organs of mice 7 days after exposure to stress

Treatment group	Spleen (mg)	Thymus (mg)	Body weight (gr)	N
Control	127.9 ± 7.3	85.8 ± 3.4	23.0 ± 0.4	6
Isolation	52.0 ± 2.6^a	21.9 ± 3.5^a	16.2 ± 1.0	6
Isolation + MLT	118.2 ± 31.3	82.0 ± 4.6^b	20.8 ± 0.6	6
Dexamethasone	66.0 ± 6.7	33.6 ± 3.7^a	15.8 ± 0.5	6
Dexamethasone + MLT	158.5 ± 14.1^b	91.3 ± 5.2^b	21.1 ± 0.6	6

MLT: 5 μg/mouse s.c., daily from day 0 until day 7. Dexamethasone: 2.5 mg/kg. i.m. on days 0, 1, 2 and 3

[a] $P < 0.05$ as compared to control group

[b] $P < 0.01$ as compared to nontreated MLT group

Table 5. Effect of melatonin on mortality of stress-exposed mice inoculated with attenuated West Nile virus (WN-25)

Treatment group	Dead/total	% of dead
Control + WN	0/12	0
Control + WN + MLT	0/12	0
Isolation + WN	12/16	75
Isolation + WN + MLT	5/16	31
Dexamethasone + WN	10/20	50
Dexamethasone + WN + MLT	5/20	25

Melatonin: 5 μg/mouse S.C. daily (2 h before darkness) from day 2 before until day 8 after virus inoculation

Dexamethasone: 2.5 mg/kg. i.m. on days 0, 1, 2, and 3

Effect of melatonin on mortality of mice inoculated with WN-25 and exposed to isolation stress or dexamethasone

The attenuated West Nile virus (WN-25) is an encephalitis virus that does not invade the brain and does not cause encephalitis but kills when injected intracerebrally [2, 14].

In a previous study we showed that exposure of mice to various stressful stimuli can induce WN-25 encephalitis [2, 5]. The purpose of these experiments was to determine if MLT can prevent this stress-induced encephalitis and death. Mice were exposed to two different stress paradigms: isolation (social), or dexamethason (pharmacological). As shown in Table 5, the stress paradigms induced mortality of 75% in isolation and 50% following dexamethasone injection. MLT administration reduced mortality rates to 31% and 25% respectively. In nonstressed mice inoculated i.p., no mortality was seen with or

without MLT. In addition, MLT prolonged the time of death in the stressed-exposed mice.

Discussion

Our results show that melatonin has a protective effect in mice infected with SFV. The drug was effective following daily s.c. injection starting 3 days before until 10 days after virus inoculation. Melatonin administration resulted in 40% to 60% mortality in comparison with 100% in the control infected mice. Moreover, MLT injection not only reduced the death rate, but significantly postponed the onset of the disease and ultimately death by 6 to 7 days. In our study we used a classic encephalitis arbovirus (SFV, alphavirus). This virus invades the CNS, and virus replication in the mouse brain eventually leads to death. Since MLT had no effect on virus growth in appropriate tissue cultures, it is conceivable that MLT affects the host resistance to the virus rather than viral replication. As reported previously [17–22], MLT might protect the mice via a peripheral immunostimulating effect. This would be consonant with the ability of melatonin to counteract the immunodepressive effect of stress exposure or glucocorticoids treatment and to protect WN-25 infected mice. This finding confirms, in a different model, the results reported by Maestroni et al. [16, 22].

In viral infections, injection of glucocorticoids caused an increase in viral titers and enhanced symptomatology and mortality [5, 13, 34]. The anti-glucocorticoid effect of melatonin might thus constitute one explanation of its antiviral activity. However, although in this study mice failed to halt viral replication after inoculation of as little as 2 to 4 PFU and ultimately died, we cannot exclude an immune-based effect of MLT on viral replication within the brain.

Melatonin has been reported to stimulate the release of interferon-γ in human and murine lymphocytes [10, 24]. Both mechanisms might contribute to the antiviral activity of melatonin. In addition, preliminary results show that the level of interferon-γ in serum of SFV infected mice was higher in MLT treated mice as compared to nontreated mice (unpubl. data).

The efficiency of MLT in protecting from lethal viral infection warrants further investigations on its mechanisms of action, its activities on the immune system, and on the possibility of using it to treat various infections and other pathologic states of the immune system.

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