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Poly I:C induced microglial activation impairs motor activity in adult rats

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Polyinosinic:polycytidic acid (poly I:C) is a synthetic double stranded RNA, which mimics with viral genome and mediates immune activation response similar to double stranded RNA virus infection into the brain. Microglial cells are the immune competent cells of the central nervous system having Toll like receptors-3 on their surface. Upon establishing that poly I:C infusion into the brain causes microgliosis by creating a viral infection model, the present study was designed to evaluate the effects of microglial activation following poly I:C infusion on motor activity. We infused 100µl of 1% solution of Poly I:C in TBE buffer directly into the lateral ventricle and TBE buffer as vehicle to controls. A significantly higher microglial cell count as compared to control on 2, 3 and 7 days post infusion was recorded. Motor activity and microglial cell count was assessed in both controls and poly I:C infused rats on 1,2,3,7,14,21 and 28 days post infusion. A significant decrease in motor activity and motor coordination occurred with respect to control. The results clearly demonstrate that microglial activation has a direct relevance with decreased motor activity. Findings could also have their importance in understanding the role of microglial cells on behavioral aspects in viral diseases.

Keywords: Microglia, Motor activity, Poly I:C, Viral infection

Immune system activation results in fatigue and decrease in physical activity in rats[1,2], mice[3,4] and in human[5]. Viral sickness model has been used to induce sickness behavior[2,6,7] and to study altered behavior[2,7-9].

Viral infection in central nervous system mainly triggers astrogliosis and microgliosis, accompanied with the increased production of proinflammatory cytokines and chemokines[10-12]. The animal models viz., chimeric murine oncovirus infection of mice[13], simian immunodeficiency virus infection of macaques[14,15], of retroviral infection mimics similar inflammatory responses. We have established that semi synthetic dsRNA, poly I:C is a potent activator of innate immune system which is mainly constituted by microglial cells in brain[16-17]. Such microglial activation produced by poly I:C infusion ultimately results in neurodegeneration that in turn further produce microglial activation[16]. The activation of microglial cells pass through a transition from resting, ramified structure to an active amoeboid state through a MHC-II expressing intermediate state[18].

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It was first reported by Alexopoulou that toll like receptor (TLR) 3 is essential for signaling dsRNA[19]. Innate and cell mediated immune activation in astrocytes and microglial cells are mediated by TLR-3 after viral exposure[20,21]. It has also been observed that Poly I:C exposed to TLR-3, present on microglial cells, mediate cell signaling to secrete proinflammatory cytokines resulting in neuroinflammation[22].

The present study has been designed to analyze the impact of induced microglial activation on motor activity in rats. Poly I:C challenged rats were used to determine whether dsRNA induced microglial activation could influence motor activity and motor coordination as earlier studies have established that Poly I:C is capable of inducing microglial activation in brain[16]. Moreover, this is a suitable model for study of neuronal changes following microglial activation. It is hypothesized that microglial proliferation and activation would impair motor activity and motor coordination. Thus poly I:C infusion in lateral ventricle of rat brain could be a suitable model for behavioral studies in relation to central glial activation.

Materials and Methods

Animals—Healthy female Wistar rats (56) weighing 200-220g, from departmental stock were

randomly selected for the present study. They were housed in controlled animal house environment (23°±1°C, 12:12h light-dark cycle) and maintained on food and water *ad libitum*. The experimental procedures were approved by the Animal Ethical Committee of the university.

Poly I:C infusion—Rats were anesthetized with ketamine (50mg/kg body wt, ip) and animals were placed in a stereotactic device (Stoelting). A cranial window was opened by drilling the area with a small burr bit, and a 26 gauge needle attached to step down Hamilton microsyringe was inserted into the right lateral ventricle (Coordinates relative to bregma: -0.8 mm anterior-posterior, +1.8 mm medial/lateral, and -3.0 mm dorsal ventral[23] . A 100 µl volume of 1% of poly I:C (Sigma Aldrich, USA) dissolved in TBE buffer was infused in rat's (n=49) right lateral ventricle at a rate of 10 µl/min with the help of Stoelting stepper motorized injector. Equal volume of vehicle (TBE buffer) at the same rate was infused in to the same site in the control (n=7) animals.

Behavioral studies

Spontaneous locomotor activity—Spontaneous locomotor activities of controls and poly I:C infused rats were monitored by computerized Optovarimax Autotrack system which is a horizontal-2D photoelectric device designed to measure behavioral activities by photoelectric beam interruption. Spontaneous locomotor activity was assessed for controls and poly I:C infused rats (7 animals/group) on 1, 2, 3, 7, 14, 21 and 28 days post infusion (DPI). Optovarimax mainly measures four major quantitative behavioral categories i.e. distance travelled (DT; actively moving distance in cm.), ambulatory time (AT, walking, circling, running etc), stereotypic time (ST; scratching, pawing, grooming, burrowing and sniffing) and resting time (RT). Only RT was calculated as time spent without beam interruption. All the control and poly I:C infused rats to be tested for open field were transferred to the test room for acclimatization. All the environmental conditions of testing room were maintained as animal house conditions to avoid environment influenced behavioral changes. The animals to be tested were placed individually in the activity monitor for 5 min just before starting the experiment and motor activity in terms of DT, RT, AT, and ST were recorded for 20 min by the software based acquisition system attached to the instrument. The monitor cage was subsequently swabbed with 100% alcohol to prevent interference from animal odors and infection. These procedures were in line with Ali *et. al*[24] and Patro *et al*[25].

Rotarod test for motor coordination—The Rotamax rotarod (Columbus Instruments; Columbus, OH, USA) was used to assess balance coordination and motor control. The apparatus consists of a motor-driven rod suspended over a grid and is capable of monitoring the performance of four rats at a time through the use of lane dividers. This apparatus is computer software based automated system which records velocity and total time spent on rod by interruption of infrared beam as rat fall off from the rod. All the vehicle (controls; n=7) and poly I:C infused rats (n=49) were acclimatized three days prior to the experiment using a constant speed (speed = 8 rpm; time=100sec.; trials=3) to familiarize them with the instrument. The animals failed in acclimatization were omitted and those succeeded were allowed to run on rotating rod (s). To assess motor coordination rotarod was used and animals were acclimatized three days prior to the experiment, with the maximum speed of 8 rpm for 100 sec. The animals succeeded in acclimatization test were allowed to run on rotating rod, scheduled 420 sec, in increment of 2 rpm/4 sec to the maximum speed of 40 rpm (trials=3). The total time spent on the rotarod (sec) was recorded for each of the three trials per rat and then averaged.

Tissue processing and immunohisto-chemistry—Same animals which were used for behavioral study were perfusion fixed for immunohistochemistry (IHC). Under deep inhalation anesthesia (diethyl ether), rats were sacrificed by transcardial perfusion with 400 ml phosphate-buffered saline (PBS) followed by 300 ml of 2% paraformaldehyde in 0.1*M* phosphate buffer (*p*H 7.4). Brain tissues having hippocampus were harvested and post fixed in the same fixative overnight at 4ºC and then cryoprotected by giving sequential changes in 10, 20 and 30% sucrose solution in 0.1*M* phosphate buffer at 4ºC. Fifteen-micrometer thick cryostat sections were cut coronally through the entire hippocampus and stored at -20ºC for further use. Five to six slides (each containing 2-3 sections) were randomly selected for Iba1 (Ionized calcium-binding adaptor molecule 1) immunolabeling using streptavidin biotin HRP method. Sections were air dried and washed in three changes of PBS (*p*H 7.4).

Membrane permeablization of sections was performed with the treatment of 0.5% Triton X-100 followed by endogenous peroxidase blocking (1% H_2O_2 in PBS) for 20 min. Non-specific proteins were blocked by incubating sections in 1% normal goat serum (NGS) in PBS at room temperature for 1 h and then incubated with rabbit polyclonal anti Iba1 antibody (WAKO, Japan) at a dilution of 1:300 (diluted in 1% BSA in PBS) at 4°C overnight. While sections used as negative control were incubated with 1% BSA in PBS only. Next day the sections were incubated with anti-rabbit biotin labeled secondary antibody from Sigma (1:100; diluted with 1% BSA in PBS) at room temperature for 1 h and further incubated with streptavidin biotin-HRP complex (RPN 1051 from Amersham) at a dilution of 1:200 in 1% BSA in PBS for 1 h at room temperature. The sections were finally visualized with 0.025% 3'3-diaminobenzidine tetrahydrochloride and 0.03% H_2O_2 in PBS for 20 min in dark. The sections were washed with water, air-dried and dehydrated in absolute alcohol. All the sections were mounted in DPX after clearing in xylene. Every time all the incubations and treatments were followed by three PBS washes of 5 min each.

Quantification and statistical analysis—Microglial cell quantification was performed with the help of Image J (V 1.38; National Institute of Health, Bethesda, MD) software. Motor coordination, spontaneous motor activity and densitometric measurements for microglial cells in poly I:C infused rats were compared with those of controls using a One-way ANOVA, followed by post hoc Tukey's test. *P* values <0.001 were claimed statistically significant. All statistical analyses were calculated using Sigma Stat statistical (Sigma Stat 3.5) analysis software.

Results

During this study adult male rats were infused with poly I:C, a semi synthetic double stranded RNA, to evaluate alterations in microglial population and associated neurobehavioral changes.

Spontaneous locomotor activity—The One-way ANOVA analysis, showed that poly I:C infused rats had decreased locomotor activity at all time points studied as compared to the controls. A highly significant decrease in DT [F(7,48) = 136; $P<0.0001$] was observed with respect to control from 1 to 28 DPI (Fig. 1a). A similar pattern of significant decrement was found for AT [F(7,48) = 22.7; $P<0.0001$ (Fig. 1b)] and ST [F(7,48) = 22.7; $P<0.0001$ (Fig. 1c)] when compared with controls. As RT has an inverse relation with DT, a significant increase was found in RT [F(7,48) = 21.4; $P<0.0001$ (Fig. 1d)] as compared to controls. A depressed locomotor activity was observed in all the cases of ST, DT, AT and RT on 1, 7 and 21 DPI. Although, a slight improvement was observed in all the locomotor activities on 2, 3, 14 and 28 DPI, but the values were not significant with respect to their respective controls.

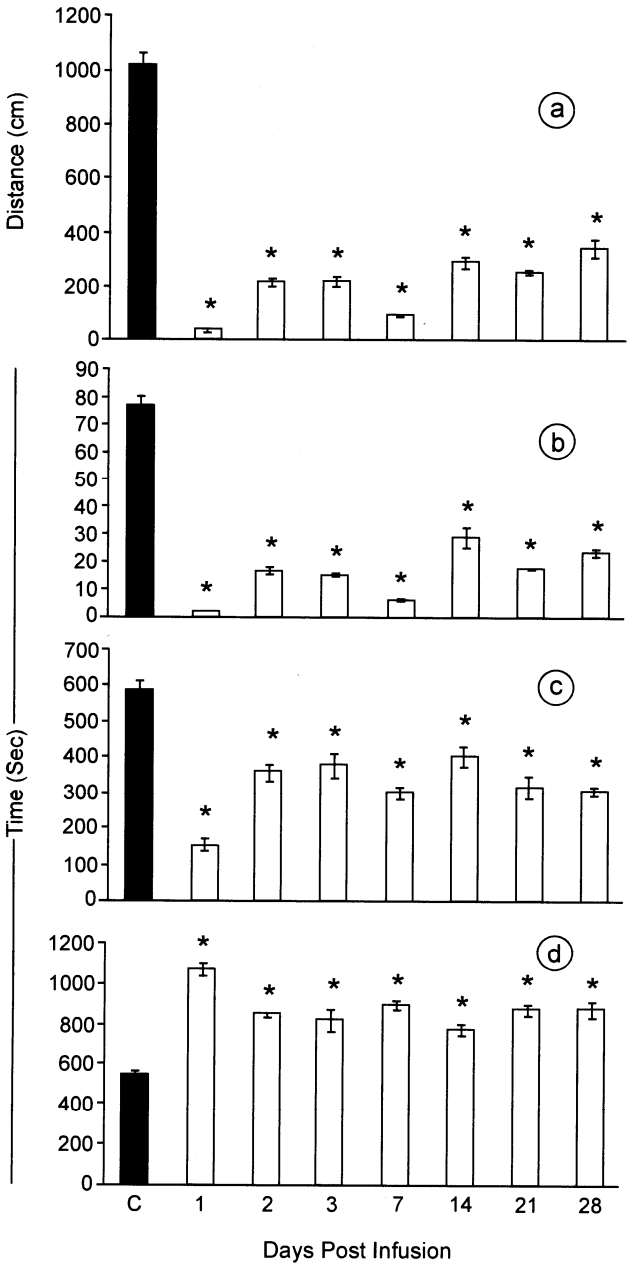

Fig. 1—Change in motor activity in vehicle treated controls and on different days post-poly I:C infusion in rats. (a) distance travelled, (b) resting time, (c) ambulatory time, (d) stereotypic time. The values are expressed as the means ± SE from 7 animals in each group.* $P<0.0001$

Motor coordination—Motor coordination was expressed as retention time on rod in an active rotarod. Poly I:C infused rats showed a poor motor coordination as compared to the controls. A highly significant decrease in retention time [F(7,48) = 27.9; $P<0.0001$] was observed in poly I:C infused rats at all time points as compared to control (Fig. 2). There was a slow decrease in rotarod performance from 1 to 3 DPI and then a gradual improvement up to 28 DPI but remains insignificant as compared to their respective controls.

Microglial activation—Microglial cell proliferation/recruitment in hippocampus was measured both in poly I:C infused and control rats. There was a significant increase in microglial cell count in hippocampus at 2, 3 and 7 day post infusion [F (7,316) =100.78; $P < 0.001$] as compared to the control (Fig. 3).

Poly I:C treatment resulted microglial activation and proliferation in the hippocampus. This alteration in microglial activation and proliferation was evident by the change in morphology (Fig. 4 a and b) and cell count (Fig. 3). Poly I:C induced microglial activation started with in 24 h and a significant increase was observed after 2, 3 and 7 DPI ($P<0.001$). The cell count started decreasing from 14 DPI onwards and by day 28, the cell number was restored to normal, approximately similar to the control values.

Discussion

The present study provide the first piece of evidence that microglial proliferation and activation after poly I:C infusion directly into the rat brain ventricle produce behavioral dysfunctions. It has been shown that a synthetic dsRNA, poly I:C infusion directly into the lateral ventricle of rat brain can induce exaggerated microglial activation and proliferation in the cerebral cortex[16]. In the present investigation neurobehavioral changes following such microglial activation were studied. Overall, the result of this study suggests that i.c.v. infusion of poly I:C

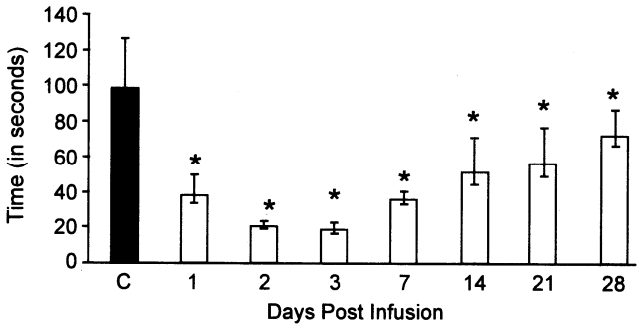

Fig. 2—Change in motor coordination as recorded by rotarod performance in vehicle treated controls and on different days of poly I:C infused rats. The values are expressed as the means ± SE from 7 animals in each group. * $P<0.0001$

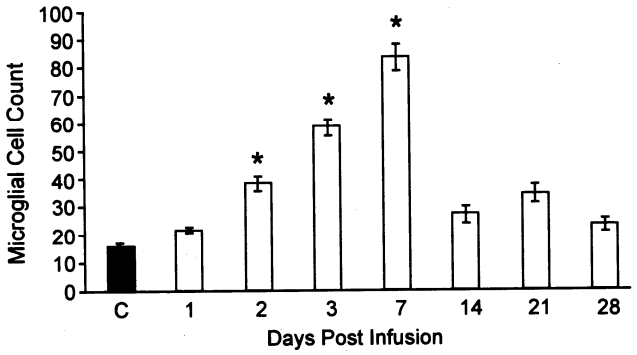

Fig. 3—Change in microglial cell count in rat hippocampus after microglial activation in vehicle treated controls and at different days post-poly I:C infusion in hippocampus. The values are expressed as the means ± SE from 7 animals in each group * $P<0.001$

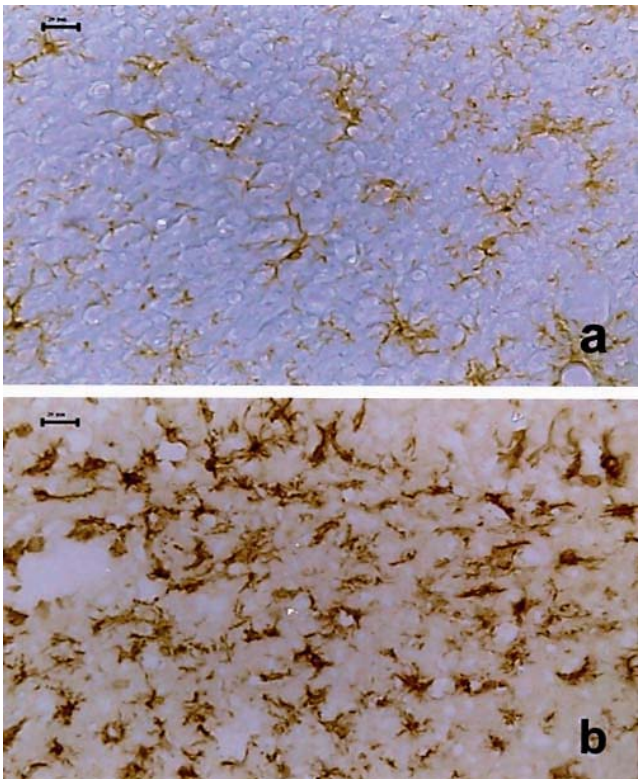

Fig. 4—Photomicrograph showing Iba1 immunostaining for microglial cells in rat hippocampus. (a) Vehicle treated control animals showing a lesser count of microglial cells with resting ramified morphology. (b) After 7days of poly I:C infusion (i.c.v.), resulted in proliferation and activation of microglial cells, presenting hypertrophied morphology characterized by short and thick processes with enlarged cell body. Scale bar =20µm.

produce subtle neurobehavioral changes due to microglial activation and proliferation. Microglia are supposed to be the first line of defense against invading pathogens entering the CNS. Many dsRNA viruses are considered as encephalitis producing retroviruses on infection[26]. In an *in vitro* study, poly I:C challenged microglial cells undergo morphological changes and become activated[27]. Poly I:C infusion in rat lateral ventricle resulted in multifold increase in microglial population and morphological transformation to various activated and phagocytic stages[16]. Activation of microglial cells results in proinflammatory cytokine and reactive oxygen and nitrogen species secretion leading to neuroinflammation[28] and shows behavioral alteration effects of infection[29,30]. It has also been studied in many infection models, primarily using postnatal rats, which also display significant behavioral abnormalities[31,32]. Zuckerman and Weiner[33] also proved that maternal immune activation by poly I:C leads to altered behavior in adult offsprings.

In the present study poly I:C induced microglial activation resulted in decreased spontaneous locomotor activity in rats. There were significantly more microglial cells in hippocampus of poly I:C infused animals. An inverse relation of proliferation with motor activity and coordination was observed. A trend of increased proliferation of microglial cells up to 7th DPI of poly I:C and then a abrupt decrease in proliferation up to 28th DPI was observed earlier[16]. Due to increased microglial proliferation, motor activity was lowest at 7th DPI and an improved motor activity was observed on 14, 21 and 28 days as the microglial cell count decreased. Similarly, in a recent study Mauricia *et. al*[34] observed that microglial cells of spinal cord in their activated state causes reduction in spontaneous locomotion. This suggests that deficit in locomotor activity after poly I:C exposure was mediated by microglial activation and proliferation. Peripheral injection of poly I:C mediated immunologically induced fatigue which resulted in lower spontaneous wheel running activity, persists for shorter duration[2,35] while i.c.v. infusion produced a longer effects on motor activity as observed in the present study.

In the present study poly I:C infusion model was used for neuroinflammation. Neuroinflammation thus induced has been recorded to cause significant decrease in locomotor activity, i.e., ST, DT and AT as well as lower motor coordination. Due to sickness behavior of these animals they present significantly high RT. Finally, it can be concluded that poly I:C mediated microglial activation has its direct effects on motor coordination and spontaneous locomotor activity. Thus this experimental model could be a useful tool in studies on pharmacology of viral diseases and cognitive changes in infective and diseased conditions.

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