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Alteration of interleukin-1α production and interleukin-1α binding sites in mouse brain during rabies infection

C. Marquette[1,2], P.-E. Ceccaldi[1], E. Ban[2], P. Weber[1], H. Tsiang[1], and F. Haour[2]

[1] Rabies Unit and [2] Pharmacology Neuro-Immuno-Endocrine Unit, Pasteur Institute, Paris Cedex, France

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Summary. We have evaluated the effect of rabies virus infection on interleukin-1α (IL-1α) production and its receptors in mouse brain. Study of virus dissemination in the central nervous system (CNS) showed a massive infection of main brain structures from day 4 post infection (p.i.) up to the agony stage on day 6 p.i. At the same time, IL-1α concentrations increased in cortical and hippocampal homogenates, whereas no change was detected in serum. In non-infected mice, IL-1α binding sites were observed in the dentate gyrus, the cortex, the choroid plexus, the meninges and the anterior pituitary. During rabies virus infection, a striking decrease in IL-1α binding sites was observed on day 4 p.i. with a complete disappearance on day 6 p.i., except in the pituitary gland where they remained at control level. In conclusion, concomitantly with the early rabid pathological signs, brain IL-1α production and IL-1α binding sites are specifically and significantly altered by brain viral proliferation. These results indicate that IL-1α could be involved in the brain response to viral infection as a mediator and could participate in the genesis of the rabies pathogeny.

Introduction

During rabies infection, immune response seems to contribute to the disease process [1]. Infection of the central nervous system (CNS) induces minor histopathological changes [2, 3], but impairment of several brain functions have been hypothesized to be at the origin of the fatal issue of the disease [4]. Rabies infection is characterized by a predominant invasion of the majority of neurons of the CNS [3–5], but with minor and inconstant cell loss [2, 5]. Rabies induces inflammation of the CNS associated with gliosis reaction and perivascular lymphocytes infiltration [for reviews see 6]. In the periphery, an overproduction of adrenocorticotropic hormone (ACTH) and glucocorticoids due to an alteration of hypothalamo-hypophyseal axis have been described [7]. This dysregulation is likely to be responsible for the alteration of the immune parameters

and the immunosuppression. These effects have been related to the critical role of cytokines produced in plasma and brain [8, 9] during viral infection.

Cytokines are key regulatory mediators involved in the host response to immunological challenge, but also in brain injury and in the communication between the immune and central nervous systems [10]. Their expression in both systems are under a tight regulatory control. However, under pathological conditions, a dysregulation may lead to an overproduction of cytokines. In this context, it has been shown that cytokines are important effectors of the inflammatory reaction of the CNS and the associated-encephalopathy (for review see [11]). One of the cytokines known to play an important role in the CNS functions and hypothalamo-pituitary-adrenal (HPA) axis regulation is the interleukin-1 (IL-1). This interleukin is important both by being a major cytokine of the immune system and by its widespread neuroendocrine action (for review see [12]). During inflammation, IL-1 produced in the brain is involved in the activation of the HPA axis by stimulation of corticotropin releasing factor (CRF) [13–15], and secretion of ACTH [16, 17] leading to suppression of the peripheral immune response [18–22]. An important role for IL-1 in brain and pituitary functions is also suggested by the presence of specific IL-1 receptors (IL-1Rs) in various structures of the mouse brain: the anterior cortex, the granular and molecular cell layers of the dentate gyrus (hippocampal formation), the choroid plexus, the meninges and the anterior pituitary [23–25]. All together, these data suggest a role for endogenous IL-1Rs/IL-1 synthesis in the brain during the host-response to infection process.

Therefore, in the present study, we have monitored IL-1α production and the modification of IL-1α binding sites in the mouse brain during the course of rabies infection, for a better understanding of the role of this cytokine in the rabies pathogenesis process.

Materials and methods

Animals

Adult male Balb/c mice (6 weeks-old) were purchased from IFFA CREDO (Lyon, France). The animals were kept under controlled light conditions in which the light period was from 07:00 am to 07:00 pm. Food and water were available ad libitum. Each mouse (3 mice per group) received bilateral inoculations (0.05 ml) of fixed rabies virus suspensions containing 5×10^7 mouse intracerebral lethal $dose_{50}$/ml ($MICLD_{50}$/ml) into the muzzle (masseter). Under these conditions, the terminal phase occurred at 6 days post-infection (p.i.).

Virus

Rabies virus CVS strain (challenge virus standard) was prepared as a 20% homogenate of infected mouse brain in saline, aliquoted and frozen at −80 °C at a viral titer of 5×10^7 $MICLD_{50}$/ml. This strain, which derives from the original Pasteur strain, was maintained in rabbit brain by successive passages. Virus titers were calculated by intracerebral inoculation into 14–16 gram Swiss mice as described by Kaplan and Koprowski [26] using the technique of Reed-Muench.

Interleukin-1α assay

Interleukin-1α concentration was measured using an enzyme linked immunosorbent assay (InterTest-1αX mouse IL-1α ELISA kit, code 1900-01: Genzyme, Cambridge, U.S.A.). The assay has a sensitivity in the range of 15 pg/ml and an intra-assay reproducibility in the range of 6.0%. Blood samples were collected at different time intervals. The serum was obtained following 5 min centrifugation at 4000 rpm, aliquoted and kept at −80 °C until use. At the same time intervals, brains were collected, and the cortices and hippocampi were dissected and homogenized at 4 °C in 1 ml of phosphate buffer saline (PBS) [$CaCl_2$ 0.7 mM, $MgSO_4$ 0.8 mM] for 30 seconds. The homogenates were centrifuged for 5 min at 10 000 rpm at 4 °C and the supernatants were aliquoted and kept at −80 °C. The assay was performed in 96-well microplates. The results were obtained by lecture on a standard scale and were expressed in pg/mg of protein after protein dosage by a Coomassie assay (Pierce).

Brain slices

Animals were sacrificed by exposure to CO_2 at day 1, 3, 4 and 6 p.i. (agony stage at day 6). Brains and pituitaries were removed, frozen on dry ice, and stored at −80 °C until use. The brains were sectioned using a cryostat (Bright, Huntingdon, UK) (15 μm thickness) and mounted on gelatin-coated slides. Brain sections were selected at level A:2 600 and A:1 500 according to a mouse stereotaxic atlas [27]. The slides were used either for IL-1α binding experiments or for staining with rabies-specific fluorescent conjugate for observation of virus-infected neurons.

Immunofluorescence

The sections were fixed in acetone (−20 °C) and stained with an antirabies nucleocapsid antibody coupled with fluorescein isothiocyanate (FITC) conjugate (Sanofi Diagnostic Pasteur, France) for 60 min, then washed in PBS and mounted in Elvanol (DuPont de Nemours, France), as described previously [28]. The sections were examined through an ultra-violet IM35 Zeiss (Iena, Germany) microscope equipped with a video-camera (Lhesa, Cergy-Pontoise, France) which allows contrast enhancing for simultaneous observation of rabies fluorescent foci and determination of anatomical structure. The microscope was further equipped with a hard-copy printer (Sony, Japan) delivering contrast-enhanced pictures of the video signal.

Binding experiments

Binding of radio-labeled human recombinant IL-1α (h^{125}I-IL-1α) was performed by quantitative autoradiography. The slide-mounted sections were preincubated 15 min at room temperature in PBS (pH 7.4), then in RPMI-1640 containing 1% of bovine serum albumin and 0.15 nM h ^{125}I-IL-1α (350 000 cpm/ml, Amersham, Les Ulis, France) at room temperature for 2 hours. Non-specific binding was assessed with an excess of unlabeled hIL-1α (10 nM) added to the incubation buffer. Following incubation the sections were washed 5 minutes in PBS buffer at 4 °C for (repeated 5 times), dried and exposed to (^{3}H) Hyperfilm (Amersham, Les Ulis, France) for 6 to 14 days. The films were processed and the relative grain density quantified by computerized densitometry using a color-coded image analyser (RAG 200, Biocom, Les Ulis, France). An internal polymer standard (Amersham ^{125}I-microscale) was used to quantify the density of receptors. The results were expressed in femtomole/mg protein, then transformed in percentage of ^{125}I-IL-1α bound in comparaison of the maximum value of ^{125}I-IL-1α bound at day 0. Each point was the mean of 6 measurements realized on each slice and with 4 slices per animal.

Results

Virus replication in the mouse brain

A progressive increase in virus titer was observed in the total brain from day 3 to day 6 p.i. (Fig. 1). Staining of tissue sections, over this time period, with an anti-rabies virus nucleocapsid antibody conjugated to FITC showed a progressive spread out of the viral infection throughout the brain. On day 3 p.i., few viral particles are detected in the cortex and thalamus (data not shown). On day 4 p.i., the cortex, the amygdala, the habenular lateral nuclei, the thalamus (ventral and ventro-lateral parts) and the hypothalamic nuclei showed viral inclusions (data not shown). On day 6 p.i. (Fig. 2), the virus was disseminated in a large number of neuronal structures: the cortical neurons were heavily infected; the striatum was also infected but the infection spared the white matter; the hippocampal formation was also well notable for the presence of inclusions in infected neurons (mainly the pyramidal layer). However, no viral inclusion was detected in the dentate gyrus and the choroid plexus.

IL-1α production in the mouse hippocampus, cortex, and plasma

The kinetics of IL-1α production in the hippocampus and the cerebral cortex showed a significant increase of IL-1α production observed from day 4 p.i. until the agony phase, on day 6 p.i., compared to IL-1α levels at day 0. The highest cytokine concentration was observed on day 6 p.i. and reached 168 and 60 pg/mg of protein in the hippocampus (3 fold increase compared to day 0) (Fig. 3A) and cortex (30 fold increase compared to day 0) respectively (Fig. 3B). In contrast, IL-1α concentration in the plasma did not exceed 5 pg/ml of serum during the whole course of the disease (data not shown).

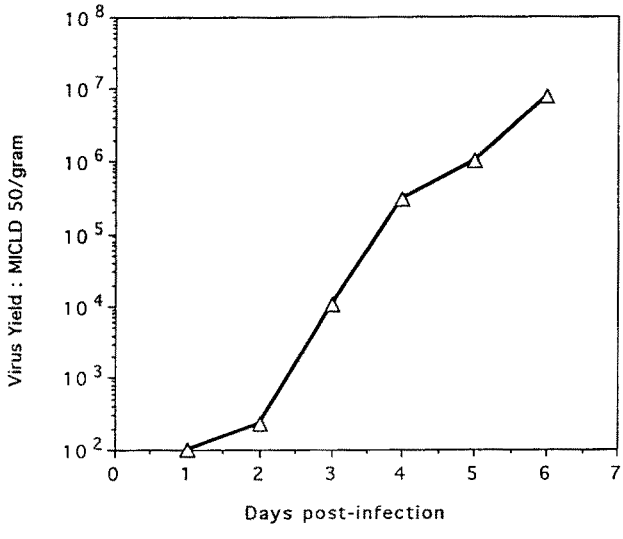

Fig. 1. Kinetics of rabies virus replication in the mouse brain cortex after intramasseter inoculation of fixed rabies virus. Results are expressed in mouse intracerebral lethal $dose_{50}$/g ($MICLD_{50}$/g) of brain cortex

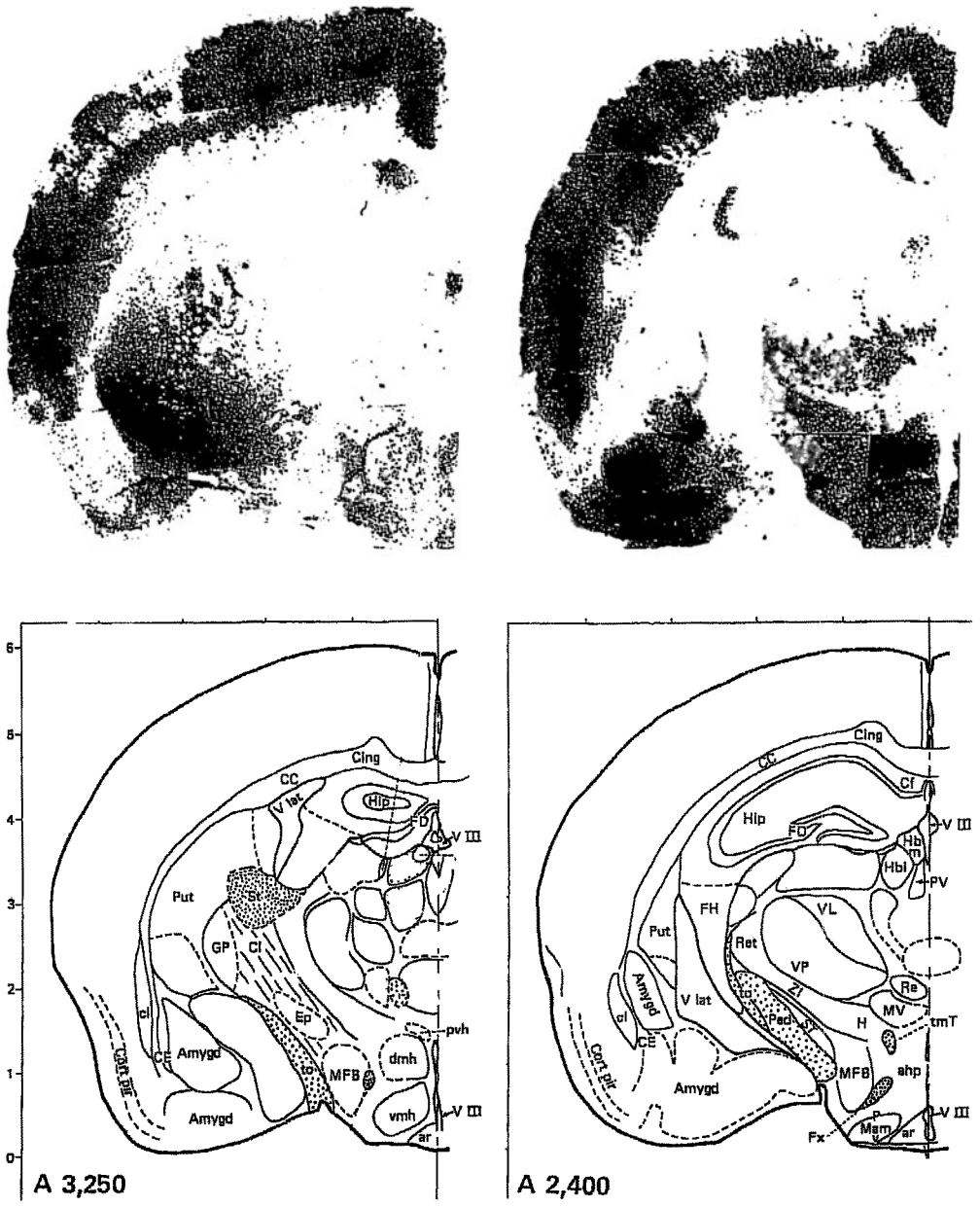

Fig. 2. Immunofluorescent detection of rabies-virus in the mouse brain at day 6 p.i. The brain structures were identified in two coronal sections at levels A:3 250 and A:2 600 according to the stereotaxic atlas of A. Lehman [27]. Abbreviations: *ahp* area hypothalamica posterior; *Amygd* corpus amygdaloidum; *ar* nucleus arcuatus hypothalami; *CC* corpus callosum; *CE* capsula externa; *Cf* commissura fornicis; *cing* cingulum; *CI* capsula interna; *cl* claustrum; *cort pir* cortex piriformis; *dmh* nucleus dorsomedialis hypothalami; *Ep* nucleus entopeduncularis; *FD* fascia dentata; *FH* fimbria hippocampi; *Fx* columna fornicis; *GP* globus pallidus; *H* area tegmentalis; *Hbl* (*Hbm*) nucleus lateralis (medialis) habenulae; *hip* hippocampus; *MFB* fasciculus medialis telencephali; *MV* nucleus medialis ventralis thalami; *Ped* pedunculus cerebri; *P Mam v* nucleus premamillaris ventralis; *PV* nucleus paraventricularis thalami; *pvh* nucleus paraventricularis hypothalami; *Put* putamen; *Re* nucleus reuniens thalami; *Ret* nucleus reticularis thalami; *ST* nucleus subthalamicus; *tmT* fasciculus mamillothalamicus; *V III* ventriculus tertius; *VL* nucleus ventralis thalami, pars lateralis; *V lat* ventriculus lateralis; *vmh* nucleus ventromedialis hypothalami; *VP* nucleus ventralis thalami, pars *posterior*; *ZI* zona incerta

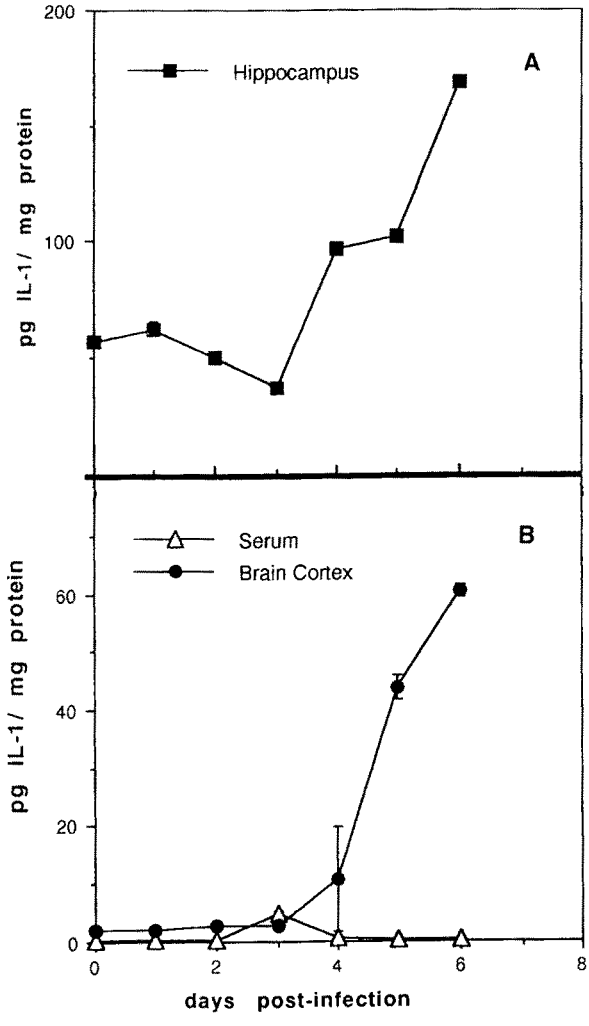

Fig. 3. Concentration of IL-1α quantified in the hippocampus and cerebral cortex homogenates of rabies virus-infected mice using ELISA assay. Results are expressed in the hippocampus (**A**) and in the cerebral cortex (**B**) in pg IL-1α/mg of protein. Error bars not shown are contained within the symbol

IL-1α binding sites modulation in the mouse brain

As indicated in Fig. 4, in uninfected control animals (day 0), only two neuronal structures exhibited binding capacity for ^{125}I-IL-1α: very intense binding was observed in the dentate gyrus whereas moderate binding occurred in the cortex. Besides these structures, ^{125}I-IL-1α binding was also observed in the non-neural tissue including the meninges, the choroid plexus and the anterior pituitary. Following infection, the normal distribution of ^{125}I-IL-1α binding was maintained up to day 3 p.i. On day 4 p.i., a strong decrease in ^{125}I-IL-1α binding densities was observed in the dentate gyrus, the choroid plexus, the meninges and the cortex. By day 6 p.i., IL-1α binding sites were undetectable in these structures. In contrast, the very high density of binding was unaffected in the anterior pituitary until the final phase of the disease (day 6 p.i.).

The image quantification analysis of the kinetics decrease in ^{125}I-IL-1α binding density was performed for the dentate gyrus and the choroid plexus

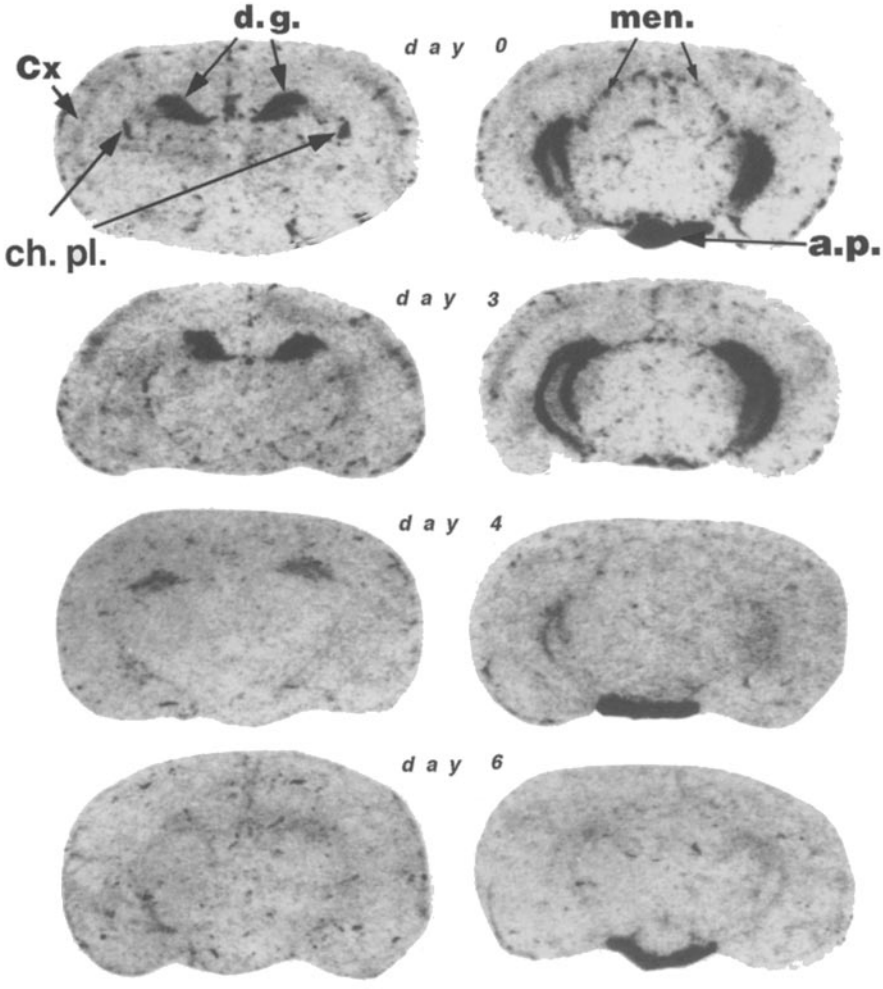

Fig. 4. Autoradiograms of ^{125}I-IL-1α binding in the mouse brain, in control (day 0) and at 3, 4, 6 days post-infection. Abbreviations: *Cx* cortex; *d.g.* dentate gyrus; *ch. pl.* choroid plexus; *men.* meninges; *a.p.* anterior pituitary

(Fig. 5): the densities of binding sites dramatically decreased between day 3 and day 4 p.i. (80%), and remained at very low levels until the final phase. In the cortex, the signal was too weak to accurately quantify variations during the different phases of infection. In the pituitary, quantification showed no significant variation in ^{125}I-IL-1α binding (data not shown).

Discussion

Our report provides evidence for the alteration of IL-1α and IL-1Rs in the mouse brain during rabies virus infection. The data suggest that they participate in the profound changes that occur during the disease [5]. A large amount of IL-1α is produced in the cerebral cortex and the hippocampus while the levels of peripheral IL-1α concentration remained low during the entire course of rabies

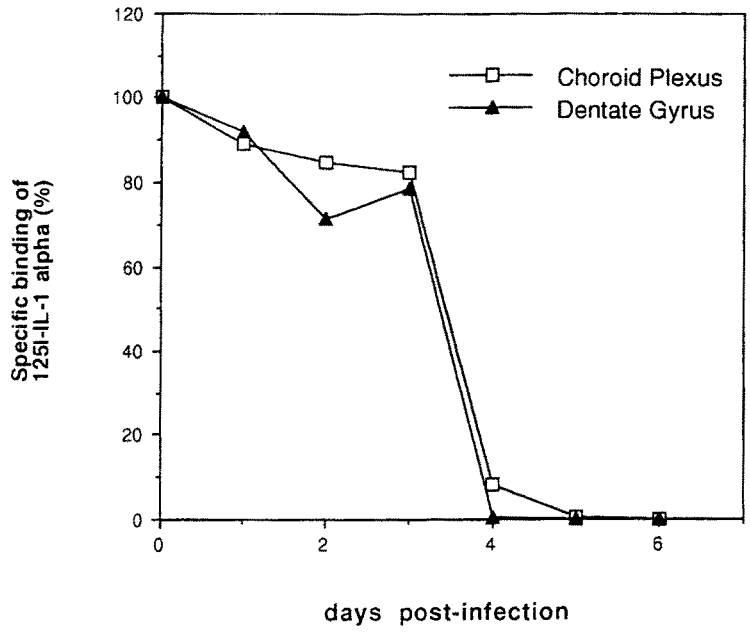

Fig. 5. Quantification of IL-1α receptors in the dentate gyrus and in the choroid plexus during the time course of rabies virus infection. Results were presented in percentage of ^{125}I-IL-1α bound in comparaison of the maximum value of ^{125}I-IL-1α bound at day 0

infection. Our data also provide evidence that IL-1α production is concurrent with an alteration of ^{125}I-IL-1α binding density in the dentate gyrus and the choroid plexus. This decrease is very significant between days 3 and 4 p.i., and with an almost complete disappearance of free receptors on the following days. On neuronal tissues, it is interesting to note that alteration of IL-1Rs and IL-1α production were not directly correlated with the presence of infectious virus particles, since no viral inclusion could be detected in the dentate gyrus and the choroid plexus at any time of the infection. Nissl histologic coloration of sections of infected mice brains displayed an intact granular and molecular cell layer of the dentate gyrus ([29] and pers. res.). Thus, the decrease in IL-1α binding sites is not due to a direct effect of the virus by either a dysregulation of their expression or by induction of neuronal cell death. These results can be compared to the modulation of IL-1α binding site densities observed in mouse brain following stimulation by a lipopolysaccharide (LPS) [24], where a "down regulation" of hippocampal IL-1 binding sites occurred [30] due to a concomitant endogenous IL-1 production [31–33]. Similar decrease in free IL-1 receptors have been also observed in street rabies and in Semliki Forest viral brain infection (Marquette and Donnelly, unpubl. data), associated with an increase in brain IL-1 messengers in this latter virus infection (Donnelly, unpubl. data). Thus, taken together these reports allow us to suggest that the alteration of free IL-1α binding sites observed during the progression of rabies, very likely results from the increase

of endogenous IL-1α and support the view that brain IL-1/IL-1Rs changes could be involved in a general response of the CNS to pathogen agent-induced inflammation.

Brain IL-1/IL-1Rs alterations could be related to the physiopathological events during the disease. IL-1 production and alteration of brain IL-1 binding sites occurred when only a few viral inclusions were present in the brain (cortex and thalamus) (day 3 p.i.), and when rabies clinical signs are not yet detectable. The greatest clinical changes occurred at day 4 p.i. (ruffled fur, cachexia, difficulty in mobility) when most of the brain areas were infected and IL-1 system already activated. Then, until death, the level of IL-1 increased and remained high concomitantly with a dramatic evolution of the clinical signs. Therefore, the early IL-1/IL-1 binding changes are not directly linked to the presence of virus in the main brain structures, but suggest that very early viral signals from other infected neuronal structures are responsible for this host response.

Important impairments of brain functions have been described in rabies pathology and our data could be included in this early neural alterations observed during rabies infection. These include modifications of neurotransmitter release (5HT, GABA) [34, 35] and their binding to receptors [36–39]. Alterations of the brain electrical activity with major changes in the regulation of sleep-wake stages have been also reported [40, 41]. Interestingly, IL-1 has been shown to be involved in several hippocampal functions including electric activities [42–44], acetylcholine release [45], and regulation of mRNA level of brain-derived neurotrophic factor [46]. Moreover, intrahippocampal administration of IL-1β in rat increased the serotoninergic metabolism locally, and the HPA axis activity with an increase of ACTH and corticosterone levels [47]. Therefore, our data are consistent with the viral induced-alterations and strongly suggest that IL-1 may be one of the factor which mediates these viral effects. Taken together, these early alterations could represent a viral mechanism for the induction of brain dysfunctions leading to the expression of the clinical/pathological manifestations before the whole brain infection.

Peripheral immunosuppression may also result from the alteration of brain IL-1 concentrations. This has been demonstrated by intracerebroventricular injection of IL-1, LPS or gp 120 (both inducing local IL-1 synthesis), inducing a marked decrease in spleen lymphocyte activity [48–50]. The cell-mediated immunosuppression induced by central IL-1 was shown to depend on the CRF release and on the HPA axis stimulation [51, 52]. This leads to the hypothesis that endogenous IL-1 produced in the brain, during rabies infection progression, may induce ACTH release leading to glucocorticoid overexpression and participate also in the peripheral immunosuppression observed, i.e. decrease in cell-mediated immunity [9, 53, 54], lymphoid depletion [55], and hypoplasia of the thymus, spleen and lymph nodes [7].

IL-1 and other cytokines (e.g., TNF, IL-6) have been also shown to participate in other virus-mediated encephalopathy and immunosuppressive diseases, i.e. during measles virus [56] or human immunodeficiency virus infections [57, 58]. Then, brain cytokines are likely to be involved in the regulatory

feedback loop leading to amplification of virus replication and in the initiation of a neurocytopathic cascade leading to brain disorders.

In conclusion, our data show that alterations of brain IL-1 and hippocampal neuron homeostasis induced by rabies infection may participate to the viral mediated-effects responsible for disrupted neuronal and immune functions. Therefore, IL-1 and other cytokines produced during virus infection may represent a general feature of neurotropic virus for the establishment of suppressive effects in host immune system.

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Authors' address: Dr. C. Marquette, Rabies Unit, Pasteur Institute, 25 rue du Dr Roux, 75724 Paris Cedex 15, France.

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