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Susceptibility to audiogenic seizure and neurotransmitter amino acid levels in different brain areas of IL-6-deficient mice

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Abstract

Interleukin-6-deficient (IL-$6^{-/-}$) mice and their normal littermate (WT) were studied to evaluate their susceptibility to seizures induced by electroshock and audiogenic stimuli at different ages. No significant changes in maximal electroshock susceptibility were evidenced between the two strains, while audiogenic seizures (AGS) can be induced only in IL-$6^{-/-}$ mice. The effects of age and genetic condition on AGSs were evaluated. The behavioural and electrocortical changes during audiogenic stimulus were observed. In addition, the levels of neurotransmitter amino acids in five brain areas (of both strains) were measured at 60 days of age. Aspartate level significantly increased in the brain stem (BS) and hippocampus (HI), while it decreased in the diencephalon (DE) of IL-$6^{-/-}$ mice. Glutamate content significantly decreased in the cerebellum (CB), DE and HI. GABA levels significantly decreased in all the areas studied. Glycine significantly decreased in the BS, CB and DE, while taurine decreased only in the DE. The levels of glutamine significantly decreased in all the areas examined, except in the cortex (CX). The changes of neuroactive amino acid levels, particularly in the BS, might explain the characteristic of high propensity to AGS of IL-$6^{-/-}$ mice. The present data support the validity of IL-$6^{-/-}$ mice as a novel epileptic model for the study of the pathophysiology and pharmacology of epilepsy.

Keywords: Audiogenic seizures; Electroshock; Epilepsy; Interleukin-6; Neuroactive amino acids

1. Introduction

Cytokines are a heterogeneous group of polypeptide mediators that have been associated classically with the activation of the immune system and inflammatory responses, but they also exert diverse actions on the peripheral and central nervous system (Hopkins and Rothwell, 1995). Various studies demonstrated that some interleukins are elevated in the cerebrospinal fluid of patients with epileptic seizures (Gidal et al., 1996; Go and Nakamura, 2002; Ichiyama et al., 1998, 2000; Kimura et al., 2002; Peltola et al., 1998, 2000a, 2000b,; Straussberg et al., 2001; Virta et al., 2002). Other reports suggested that interleukin-6 (IL-6) appears to be involved in excitotoxicity-induced brain damage (Ali et al., 2000; De Bock et al., 1996; Gadient and Otten, 1997; Higuchi et al., 1994; Hopkins and Rothwell, 1995; Minami et al., 1991). In addition, in vitro studies showed that IL-6 can significantly protect against glutamate- and NMDA-induced excitotoxicities (Ali et al., 2000; Carlson et al., 1999; Toulmond et al., 1992; Yamada and Hatanaka, 1994).

In the mouse, various single-gene mutations that cause epilepsy were identified (Noebels, 1984; Seyfried and Glaser, 1985), and it was hoped that the identification and

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study of these genes might provide insights into human epilepsy (Engel, 1995; Gale, 1995; Misawa et al., 2002; Noebels, 2003). Although the epileptic manifestations may be logically correlated with the developmental abnormalities of the neocortex, the hippocampus and, perhaps, other brain areas, their neuropathophysiological mechanisms remain to be better elucidated. Susceptibility to audiogenic seizure (AGS) varied widely between the inbred strains of mice (Neumann and Collins, 1991). The laboratory-selected IL-$6^{-/-}$ mice might represent an interesting animal model for investigating the role of IL-6 in the physiological and pathological processes (Poli et al., 1994).

The present work was directed toward the evaluation of IL-$6^{-/-}$ mouse susceptibility to seizures. This was accomplished by the determination of susceptibility to AGSs and to maximal electroshock and by measuring the levels of neurotransmitter amino acids in different brain areas. The biochemical data and the convulsant phenomena observed in IL-$6^{-/-}$ mice were correlated with those of wild-type (WT) mice. The latter strain of mice was chosen as it represents the normal littermate (Poli et al., 1994).

2. Materials and methods

2.1. Animals

WT and IL-$6^{-/-}$ mice were originally obtained from IRBM "P. Angeletti" (Pomezia, Italy) and then maintained at the Institute of Pharmacology, University of Catanzaro (Italy). The chimeras, from which both strain of mice were derived, were obtained from two cell lines transmitted the mutation to their progeny, derived from the C57BL6/DBA/2J (B6/D2 hybrid; Poli et al., 1994). The animals were maintained under environmentally controlled conditions (7 a.m.:7 p.m. light/dark cycle, 22–24 °C, food and water available ad libitum). The procedures involving animals and their care were conducted in conformity with national and international laws and policies [EEC Council Directive of 24 November 1986 (86/609EEC)]. All animal experiments were carried out according to the NIH animal care guidelines (NIH Publication No. 80-23). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Mouse production and genotyping, generation and genotyping of mutant mice and probes and in situ hybridization were previously described by Poli et al. (1994).

2.2. Induction of AGSs

The mice were placed into a sound-attenuating chamber equipped with a glass door for observation. Each mouse was placed under a hemispheric perspex dome (diameter 58 cm) and, for 2 min, was allowed for habituation and assessment of locomotor activity. Auditory stimulation (12–16 kHz, 109 dB) was applied for a maximum of 1 min or until tonic extension occurred. Both the groups of mice were tested at ages 10, 14, 18, 22, 26 30, 35, 45 and 60 days because AGS susceptibility is age dependent. None of the mice had previous exposure to acoustic stimulation. The sample size for each group was from 8 to 18 mice. Each mouse was used only once.

The intensity of response was categorized as previously described (De Sarro et al., 1984): no response (0); wild running (1); clonic seizure (2); tonic seizure (3); respiratory arrest (4).

2.3. Maximal electroshock

The maximal electroshock seizure threshold (MEST) for the tonic extension of the forelimbs were determined according to the method previously described by Swinyard and Woodhead (1982). Briefly, seizures were produced by using ear-clip electrodes and by alternating current delivered by a stimulator, with a stimulus duration of 0.2 s and from 5 to 50 mA. Tonic hind-limb extension was taken as the end point of MESTs. The convulsive threshold was evaluated as CD_{50}, which is a current strength (in mA) necessary to produce tonic hind-limb extension in 50% of the tested animals. To estimate the convulsive threshold, at least four groups of mice (8 to 10 animals per group) were challenged with electroshocks of various intensities. Subsequently, an intensity–response

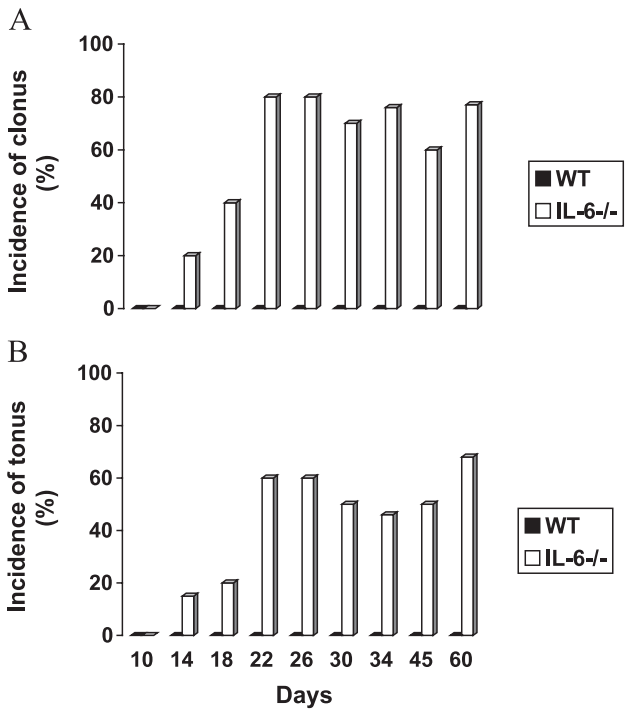

Fig. 1. Audiogenic seizure response in WT and IL-$6^{-/-}$ mice. The abscissae shows the age of animals and the ordinate shows the incidence of clonic (A) and tonic (B) seizures.

Table 1
Maximal electroshock susceptibility in WT and IL-6$^{-/-}$ mice at different ages

Age (days)	CD_{50} value (confidence limits)	
	WT	IL-6$^{-/-}$
21	6.0 (5.00–7.22)	6.1 (5.00–7.44)
28	6.5 (5.50–7.97)	6.6 (5.75–7.58)
35	7.6 (6.20–9.32)	7.7 (6.30–9.41)
42	8.8 (7.00–11.06)	8.4 (6.90–10.2)
60	9.4 (8.00–11.07)	9.0 (7.80–10.4)

Data, expressed as mA, were calculated according to the method of Litchfield and Wilcoxon (1949).

curve was calculated on the basis of the percentage of mice convulsing.

2.4. Electroencephalographic recordings

The electroencephalographic activity recordings were performed as previously described (De Sarro et al., 1997). Briefly, five steel-screw electrodes were chronically implanted, under fluothane anaesthesia, bilaterally onto the frontoparietal areas. The correct position of the electrodes was confirmed by anatomical analysis.

2.5. Measurement of amino acid levels

Amino acid levels were determined in the brain stem (BS), cerebellum (CB), cortex (CX), diencephalon (DE) and hippocampus (HI) of IL-6$^{-/-}$ and WT mice at 60 days of age. Supernatants from the homogenates of the brain areas studied were deproteinized by ultrafiltration, and 50-μl aliquots of ultrafiltrate were dried. Amino acids were derivatized with phenylisothiocyanate, and their content was determined by HPLC in a waters chromatografic system equipped with a Pico-Tag column (3.9×300 mm) containing a high-efficiency reverse-phase silica packing.

2.6. Statistical analysis

The statistical comparison between the groups of IL-6$^{-/-}$ and WT mice was made using Fisher's exact probability test (incidence of the seizure phases). The percentage incidence of each phase of the AGS was determined for each strain of mice. The percentages of animals exhibiting clonic or tonic seizures following different current intensity stimuli were calculated, and these values were plotted against corresponding doses for the calculation of CD_{50}, with 95% confidence limits, as described by Litchfield and Wilcoxon (1949). At least 32 animals were used to calculate each CD_{50} value.

The SAS statistical package for personal computer was used to analyze biochemical data (SAS Institute, 1987).

3. Results

3.1. AGSs

In our initial experiments, when responsiveness to sensory stimuli was tested, we realized that a loud sound can induce seizures in IL-6$^{-/-}$ but not in WT mice. Seizure in IL-6$^{-/-}$ mice to loud (109 dB) sound started within 20–30 s, with wild running followed by erratic leaping and clonic convulsions that, 40–50 s from the beginning of the stimulus, culminated in tonic hind-limb extension, which, in a few occasions, was followed by respiratory arrest and death. Seizures in IL-6$^{-/-}$ mice were not readily induced by handling.

The manifestation of AGSs in IL-6$^{-/-}$ mice was age dependent (Fig. 1). The animals showed no convulsions at the age of 10 days. At all ages studied, wild running was evident in 90% of the mice, and, approximately at 14 days, 67% of the IL-6$^{-/-}$ mice displayed clonic and tonic

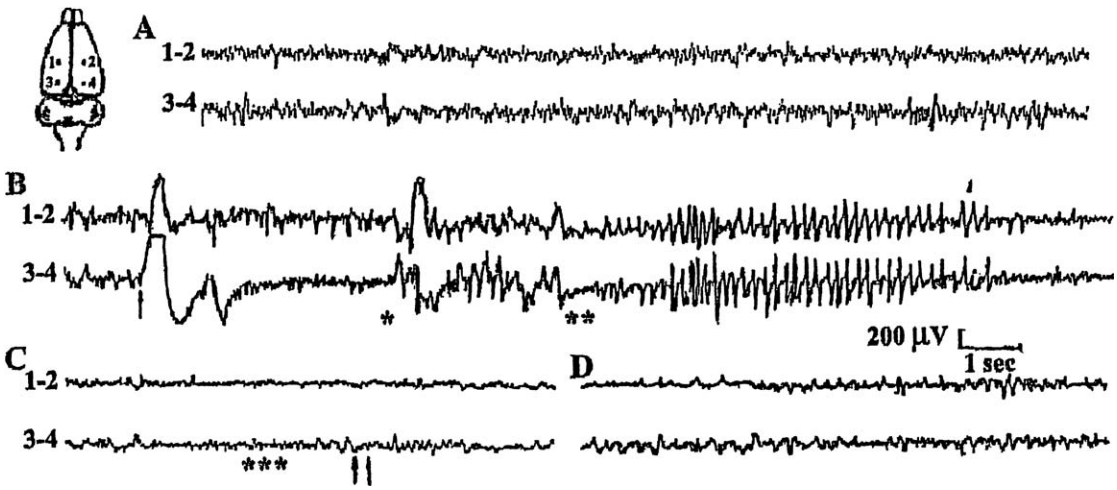

Fig. 2. Electrocorticographic patterns from the right and left frontal (1–2) and parietal (3–4) cortex, illustrating the effects of the audiogenic stimulus in (A) IL-6$^{-/-}$ mice and (B) control. ↑ Onset of sound stimulation; *beginning of the running phase; **beginning of chronic and tonic phases; (C) end of the tonic phase and beginning of the postictal period; ***end of hind lemb extension; (D) postictal period (EcoG activity 80 to 90 s after the seizure onset).

seizures following audiogenic stimulation. Respiratory arrest was evident in less than 30% of the animals from 18 to 30 days. WT mice age-matched control never displayed clonic and tonic seizures, although wild running was observed from 18 to 35 days of age (40–70%). AGSs were not simply due to increased stress responsiveness to loud sound because a shorter (40 s) 83-dB stimulus did not elicit an AGS in IL-$6^{-/-}$ mice.

3.2. Maximal electroshock

Table 1 shows the MEST values (mA) and CD_{50} values for WT and IL-$6^{-/-}$ mice. No significant differences in MEST values were observed between the WT and IL-$6^{-/-}$ mice. IL-$6^{-/-}$ mice show only a weak reduction of CD_{50} values in comparison with WT mice at 42 and 60 days of age.

3.3. Electrocortical activity

The electrocortical activity following AGS is reported in Fig. 2. In particular, ECoG was usually characterized by sharp- or spike-waves, followed by polyspikes and epileptiform discharges that appeared in IL-$6^{-/-}$ but not in WT mice.

3.4. Neurotransmitter amino acid levels

A significant increase of aspartate content was found in the BS and HI, while a significant decrease was evidenced in the DE of IL-$6^{-/-}$ mice in comparison with WT mice (Table 2). Glutamate content was found significantly decreased in the CB, DE and HI (Table 2). Levels of glutamine, a nonneurotransmitter amino acid, were also determined for the metabolic relationship among glutamine, glutamate, aspartate and GABA. They were found to be significantly decreased in all the areas examined, except at CX (Table 2). GABA levels significantly decreased in all the five areas studied (Table 3), where the maximum level of significance was observed in the CX and CB. Glycine was found to be significantly decreased in the BS, CB and DE, and a decrease of taurine content was observed only in the DE (Table 3).

Table 2
Aspartate, glutamate and glutamine levels in different brain areas of WT and IL-$6^{-/-}$ mice

Amino acid	Brain area	WT (n=10)	IL-$6^{-/-}$ (n=5)	P
Aspartate				
	Cortex	4.66±0.20	4.59±0.57	.887
	Hippocampus	3.63±0.37	5.35±0.51	.018
	Diencephalon	6.71±0.87	4.55±0.09	.043
	Brain stem	3.42±0.33	5.46±0.25	.001
	Cerebellum	6.61±0.31	6.28±0.18	.492
Glutamate				
	Cortex	3.02±0.15	2.94±0.23	.775
	Hippocampus	3.53±0.59	0.99±0.13	.011
	Diencephalon	5.62±0.41	1.37±0.16	.000
	Brain stem	1.73±0.29	1.76±0.22	.947
	Cerebellum	6.63±0.78	2.80±0.12	.005
Glutamine				
	Cortex	2.03±0.20	1.95±0.48	.857
	Hippocampus	1.46±0.18	0.19±0.07	.000
	Diencephalon	2.65±0.34	0.21±0.08	.000
	Brain stem	1.84±0.21	0.85±0.21	.011
	Cerebellum	1.94±0.19	0.34±0.08	.000

Data, expressed as μmol/g wwt, are mean±S.E.M. Column P reports statistical differences.

Table 3
GABA, glycine and taurine levels in different brain areas of WT and IL-$6^{-/-}$ mice

Amino acid	Brain area	WT (n=10)	IL-$6^{-/-}$ (n=5)	P
GABA				
	Cortex	6.88±0.36	4.44±0.33	.000
	Hippocampus	4.86±0.33	2.75±0.30	.001
	Diencephalon	9.46±1.05	4.85±1.16	.018
	Brain stem	7.54±0.21	6.06±0.21	.000
	Cerebellum	7.65±0.32	3.78±0.48	.000
Glycine				
	Cortex	2.62±0.19	2.40±0.06	.434
	Hippocampus	2.28±0.18	2.03±0.25	.430
	Diencephalon	5.42±0.57	3.15±0.45	.022
	Brain stem	5.19±0.54	3.20±0.24	.026
	Cerebellum	3.56±0.26	2.44±0.12	.012
Taurine				
	Cortex	6.07±0.28	6.55±0.34	.325
	Hippocampus	4.58±0.40	4.57±0.41	.988
	Diencephalon	5.33±0.66	2.76±0.41	.022
	Brain stem	3.35±0.22	3.07±0.13	.414
	Cerebellum	5.91±0.23	5.92±0.22	.979

Data, expressed as μmol/g wwt, are mean±S.E.M. Column P reports statistical differences.

4. Discussion

Both strains of mice were tested during the first 60 days of age, when WT mice were resistant to sound-induced seizures. No clear differences were observed between the IL-$6^{-/-}$ and WT strains of mice to MEST susceptibility.

If we compare the present data with those of the genetically epilepsy-prone DBA/2J mice that were found to exhibit a genetic susceptibility to AGSs (Hall, 1947), we will observe that audiogenic susceptibility is an age-dependent phenomenon in both strains, but the pattern is different. In DBA/2J mice, it begins between 14 and 16 days, is maximal at 22–26 days and is absent at age 42 or 80 days (Chapman et al., 1987; Engstrom and Woodbury, 1988). In IL-$6^{-/-}$ mice, AGSs have a maximal incidence at 22, 26 and, also, at 60 days of age. On the other hand, our wild-type strain IL-$6^{+/+}$ showed no AGS susceptibility from 14 to 60 days of their life. These data concerning AGS and maximal electroshock susceptibility are partially in agree-

ment with previous reports (Engstrom and Woodbury, 1988; Engstrom et al., 1986).

When we have compared maximal electroshock susceptibility in WT and IL-6$^{-/-}$ mice, we noted that with age, MEST decreased in both strains, and that at 42 and 66 days, IL-6$^{-/-}$ appeared to be more susceptible than WT mice were (see Table 1), even if nonsignificant differences between the two strains were evident. The present data are in agreement with those recently described by our group in 4-aminopyridine-induced seizures, an experimental model that is, in part, similar with maximal electroshock (De Sarro et al., in press), and suggest that voltage-dependent ionic mechanisms are not significantly affected in IL-6$^{-/-}$ mice. In addition, the excessive excitatory amino-acid-mediated synaptic driving, recently observed by our group (De Sarro et al., in press), may have led to a hyperexcitable condition that is responsible for the AGS susceptibility occurring in IL-6$^{-/-}$ mice.

It was reported that the genes on chromosomes 4, 7, 12 and X are involved in AGS susceptibility (Brennan et al., 1997; Malas et al., 2003; Misawa et al., 2002; Musumeci et al., 2000; Neumann and Collins, 1991; Neumann and Seyfried, 1990; Noebels, 2003; Rise et al., 1991; Seyfried and Glaser, 1985; Upton and Stratton, 2003). The present results clearly show that gene manipulation is responsible for brain abnormalities, which may be associated with epileptic seizures (Poli et al., 1994) and a higher seizure susceptibility to audiogenic stimulus (present data).

The excitatory system, mainly consisting not only of glutamate (Fonnum, 1984) but also aspartate (Gundersen et al., 1998), was affected in IL-6$^{-/-}$ mice. In particular, a highly significant increase of aspartate was observed in the BS, where acoustic pathway is located. Glutamate concentration was unchanged in the CX and BS, while a decrease was evidenced in the HI, DE and CB. This decrease in glutamate content is a paradox because seizures are generally associated with increased glutamatergic activity. However, it is only the glutamate in the synaptic cleft that can activate the receptors, and it could be that this glutamate is not changed. It is well known that glutamate is released from glutamatergic neurones and is taken up by astrocytes to be released as glutamine after its conversion by glutamine synthetase, which has a glial localization (Martinez-Hernandez et al., 1977; Tansey et al., 1991). This forms the basis for the so-called glutamate–glutamine cycle (Van den Berg and Garfinkel, 1971). Therefore, the decrease in glutamate content observed in the HP, DE and CB could, in part, explain the decrease in glutamine content. In the BS, the decreased amount of glutamine and the unchanged level of glutamate could indicate a lower "trafficking" of glutamate in the nonneuroactive form (glutamine). The significant decrease of GABA, evidenced in all the brain areas studied, could be responsible of AGS, as previously suggested (Engstrom and Woodbury, 1988; Kash et al., 1997; Sykes and Horton, 1982). In addition, the excessive aspartate content in the BS, where the acoustic pathway is located, might have led to a hyperexcitable condition that is responsible for the epileptic manifestations occurring in IL-6$^{-/-}$ mice. Both changes (i.e., GABA decrease and aspartate increase) could be responsible for the increased susceptibility to kainic-acid-induced seizures recently reported by Penkowa et al. (2001). The reduction of glycine levels in some brain areas was considered to be responsible for the increased susceptibility to AGS in DBA/2J mice (Engstrom and Woodbury, 1988) and could participate to the AGS occuring in IL-6$^{-/-}$ mice. We do not know if other brain abnormalities in IL-6$^{-/-}$ mice may induce a higher susceptibility to AGSs.

Our data showed an apparent variance with the findings of Campbell (1998), Campbell et al. (1993), Chiang et al. (1994) and Samland et al. (2003). These authors showed that the mice with a chronic production of IL-6 and glial fibrillary acid protein have an impaired blood–brain barrier functionality, spontaneous behavioural seizures, ataxia, increased hippocampal excitatory activity (Steffensen et al., 1994) and neurodegeneration. Our animals did not show spontaneous seizures, and, similar with those of Penkowa et al. (2001), no neurodegenerative changes were observed during the first year of their life.

Additional studies could be done in IL-6$^{-/-}$ mice to better characterize whether their seizure susceptibility depends on similar mechanisms already described in DBA/2J mice. It will be of interest to compare IL-6$^{-/-}$ mice with similar models that lack or overexpress other cytokines to assess the relative epileptic potential of cytokines and to help the identification of potential targets for therapeutic interventions.

In conclusion, our findings indicate that IL-6$^{-/-}$ mice show a range of biochemical and behavioral changes, correlated, in particular, with a higher AGS susceptibility.

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