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Research report

Relation of behaviour and macrophage function to life span in a murine model of premature immunosenescence

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Abstract

According to our previous work, mice of the same strain and age show striking inter-individual differences in behaviour when exposed to a T-maze test. Further, the animals exploring the maze slowly (slow mice) or staying at the starting point (*freezing behaviour*), which show high levels of emotionality/anxiety in other standard behavioural tests, have a less competent immune system (earlier immunosenescence) than those which explore it quickly (fast mice). The present longitudinal study on OF-1 Swiss female mice confirms and extends the above findings. Thus, the animals showing a lower performance in the T-test (slow mice) which is accompanied by a poor neuromuscular coordination in a tightrope test, have a shorter life span than the good performers (fast mice). Moreover, the slow mice have a less competent immune system as regards the following functions of peritoneal macrophages: adherence to substrate, chemotaxis, ingestion of particles and superoxide anion production. This suggests that, at the same chronological age and as regards their immune competence, the slow mice are *biologically older* than the fast mice. This agrees with current ideas on the close functional relationship between the nervous and the immune system in the physiological adaptation to stress, and supports the concept that an optimum level of performance of these two systems is needed to attain a long life span.

Keywords: Life span; Behaviour; Macrophage; Mice; Biological age; Neuro-immune communication

1. Introduction

It is generally accepted that two key integrating systems, i.e. the nervous and the immune system, are closely interconnected at the cellular level. These systems express a large number of regulatory molecules and respond to them, and in fact, cytokines, hormones and neurotransmitters as well as their receptors, are present in both the brain and the immune system. Moreover, the pattern of synthesis of these molecules is similar in nervous cells and in leukocytes [4,12,17]. Ageing is associated with a decline of several functions, including those of the nervous system such as learning and memory [8], motor function and emotional behaviour [4,5], and the immune system [31,38,37]. Moreover, a disrupted bidirectional communication between these two systems may lead to the loss of homeostasis that plays a fundamental role in age-related physiological decline [18,20] enhancing the probability of death. Undoubtedly, some processes to which aged people are particularly prone such as neurodegenerative and inflammatory diseases, like Alzheimer and Parkinson disease include a failure in neuro-immune interaction in their pathology [46].

From both a theoretical and a practical viewpoint it is very important that the nervous–immune network is involved in the adaptation to all types of stressful stimuli and that an inadequate response to environmen-

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tal stressors has been linked to a dysfunction of the nervous–immune interaction [17,42]. Moreover, it has been proposed that stress accelerates the process of biological ageing and that senescence is accompanied by an impaired stress response [16,45].

Several studies [39,15] have shown a relation between hyper-reactivity to novel environmental stimuli in an exploration test and life span. More specifically the data indicate that the life span of inbred rodent strains is inversely related to the intensity of their behavioural responses to stressful stimuli. Moreover, according to Gilad and Gilad [19], the shorter life span of the hyper-reactive animals could be caused by an accelerated age-dependent functional decline in specific stress-responsive neuronal systems.

Behavioural and immunologic tests have been major components in batteries designed to assess biological ageing in animal and human populations. In agreement with this, we carried out previous cross-sectional studies on aged [14] or adult [9] female Swiss mice, that showed interindividual differences in a simple T-maze test. These studies also revealed that animals which took longer to explore the maze or showed the 'freezing behaviour' that usually accompanies high levels of anxiety ('slow mice') had an impaired immune function in comparison to the 'fast mice' (that explored the first arm of the T-maze in 20 s or less). Therefore, the slow mice were proposed as a model of premature immunosenescence, and in order to test the hypothesis that at the same chronological age the slow mice are indeed 'biologically older' than the fast mice, we have investigated their behaviour using additional methods. Thus, our recent data [42] demonstrate that the slow mice have a low muscular coordination and vigor in comparison to the fast mice, as assessed by a tightrope test [33] similar to that used by Ingram [24] for behavioural determination of biological age in the laboratory mouse. Moreover, these slow animals show a decreased locomotor activity in stressful situations and a less adaptive response to stress in three standard behavioural tests (the holeboard, the open field and the plus-maze tests) when compared to fast mice [43]. In addition, several functions of macrophages and lymphocytes, which decrease with age, were more impaired in the slow animals [43]. In view of the fact that ageing results in an alteration of the neuro-immune interactions with resulting cognitive and immune function decline, changes in these functions can be considered biomarkers of physiological age and therefore the present model of premature immunosenescence may be useful in gerontological research.

Our previous cross-sectional studies of interindividual differences in age-related nervous–immune decline, are complemented here by a longitudinal research. This is justified since, as pointed out by Dellu et al. [15], "Ageing is a continual process starting in embryo, which can thus be analyzed in a developmental perspective based on data from multiple time points (longitudinal analysis)… Over a life time, alterations observed in the elderly could well be related to factors detectable early in life". In agreement with this view, the experiments presented below were performed in order to confirm that a deficient (slow) performance in the T-maze test is generally predictive of early immune decline and short life span.

2. Materials and methods

2.1. Animals

We have used female OF-1 Swiss mice (*Mus musculus*) (Harlan Ibérica, Barcelona, Spain), which were 6 weeks old on arrival to our laboratory. The mice were specific pathogen free, as tested by Harlan according to FELASA recommendations. The animals were randomly divided in groups of five, and each group was housed in polyurethane boxes, at a constant temperature (22 ± 2 °C) in sterile conditions inside an aseptic air negative-pressure environmental cabinet (Flufrance, Cachan France) on a 12/12 h reversed light/dark cycle. All animals were fed water and standard Sander Mus (A. 04 diet from Panlab L.S. Barcelona, Spain) pellets ad libitum. The diet was in accordance with the recommendations of the American Institute of Nutrition for laboratory animals.

2.2. Experimental groups

The animals were marked for their individual follow-up. At 16 ± 2 weeks of age the spontaneous exploratory behaviour of each mouse was tested in a T-shaped maze. This apparatus essentially consists in three arms made of wood covered in their internal face by black metacrilate. The inside dimensions of every arm are 10 cm wide, 25 cm long and 10 cm high. The floor is made of cylindrical aluminum rods 3 mm thick placed perpendicularly to the side walls. The test is performed holding the mouse from the tip of the tail and placing it inside the 'vertical' arm of the maze with its head facing the end wall. The performance is evaluated by determining with a chronometer the time elapsed until the animal crosses with both hindlegs the intersection of the three arms. This test was performed four times, once every 15 days, in order to sort out the 'fast' mice (which complete the exploration of the first arm of the maze in 20 s or less) from the 'slow' mice (which require over 20 s). Then, the animals were distributed in two groups. One group of 18 mice contained the fast population and the other, of 12 mice, the slow population with a fast/slow mouse ratio of 100/0 and 0/100, respectively. Immediately after being tested in the T-shaped

maze, the animals were subjected to the tightrope test for evaluation of neuromuscular coordination and vigor (21). These tests were performed always between 10:00 and 12:00 h and under red light.

2.3. Tightrope test

In the tightrope test the mouse is placed on the middle of a tightrope tied up on each side to the rod of a chemical stand. The tightrope, which is 60 cm in length, is suspended above a mouse cage, at about 40 cm of its bedding of wood shavings. The mice have to reach one end of the side poles. Only 1 min is allowed for completion of the test. The time in seconds (until contact with the pole is made by the head, body or limbs) is recorded and the animal is scored positive. Failure to reach the pole either by falling or by not progressing enough towards the sides in the allowed time of 1 min is scored negative. The data have been shown as the percentage of mice completing the test.

After the tightrope test performance the mice were weighted. A follow-up of body weight was carried out with age, from 6 to 18 months of age.

2.4. Collection of peritoneal leukocytes

In this longitudinal study, the peritoneal suspensions were obtained monthly without sacrificing the mice. The mice were held by the cervical skin, the abdomen was cleansed with 70% ethanol, and 3 ml of sterile Hank's solution was injected intraperitoneally. After massaging the abdomen, 80% of the injected volume was recovered. Macrophages, identified by morphology and non-specific esterase staining, were counted and then adjusted by dilution with Hank's solution to 5×10^5 macrophages/ml. The cellular viability, determined in each experiment using the trypan-blue exclusion test, was in all cases higher than 95%.

2.5. Assay of phagocytic function in peritoneal macrophages

The study of different steps of the phagocytic process, i.e. adherence to tissues, mobility to infectious focus (chemotaxis), phagocytosis and superoxide anion production was carried out on the peritoneal suspensions, with macrophages being adjusted to 5×10^5 cells/ml Hank's.

For the quantification of adherence capacity to the substrate, we observed the adherence to a smooth plastic surface, because it resembles adherence to animal tissue. The method was carried out as previously described by De la Fuente et al. [13]. Briefly, aliquots of the peritoneal macrophage suspensions (adjusted) were placed in eppendorf tubes and incubated 10 min at 37 °C, and after shaking gently, the number of non-adhered macrophages was determined in Neubauer chambers. The adherence index, AI, was calculated according to the following equation:

$$\mathrm{AI} = 100 - ((\text{non-adherent cells/ml})/(\text{initial cells/ml})) \times 100$$

The chemotaxis assays were performed according to a modification [13] of the original technique described by Boyden [6], which consists essentially in the use of chambers with two compartments separated by a filter (Millipore, Bedford, MA) with a pore diameter of 3 μm. Aliquots of 0.3 ml of the peritoneal suspension (5×10^5 cells/ml) were deposited in the upper compartment of the Boyden chambers. F-met-leu-phe (Sigma, St. Louis, MO) (a positive chemotactic peptide in vitro), at 10^{-8} M, was placed in the lower compartment in order to determine chemotaxis. The chambers were incubated for 3 h at 37 °C and 5% CO_2, and after this time the filters were fixed, stained and the chemotaxis index (CI) was determined by counting in an optical microscope (immersion objective) the total number of macrophages in one third of the lower face of the filters.

The latex phagocytosis assay was carried out following the method described by De la Fuente [11]. Aliquots of 200 μl of peritoneal suspensions were incubated in culture plates (Sterilin, Teddington, England) for 30 min. After being washed with PBS (phosphate buffer saline), 20 μl latex beads (1.09 μm diluted to 1% PBS, Sigma, St. Louis, MO) were added to the adherent monolayer. After 30 min of incubation, the plates were washed, fixed and stained and the number of particles ingested by 100 macrophages was counted. The results were expressed as phagocytic index (PI).

Superoxide anion production, the first response in the respiratory burst, was evaluated assessing the capacity of this anion, produced by macrophages, to reduce nitroblue tetrazolium (NBT). This was carried out following the method described by De la Fuente et al. [13] slightly modified as follows. Aliquots of 250 μl of peritoneal suspension were mixed with 250 μl of NBT (1 mg/ml in PBS, Sigma), 50 μl of a latex bead suspension were added to the stimulated samples and 50 μl of PBS to the non-stimulated samples. After 60 min of incubation, the reaction was stopped, the samples were centrifuged, and the absorbance of the supernatants was determined at 525 nm in spectrophotometer (extracellular measure of superoxide anion production). The intracellular reduced NBT was extracted with dioxan (Sigma) and, after centrifugation, the supernatant absorbance at 525 nm was determined (intracellular superoxide anion production). The results were expressed as nmol/10^6 cells using a pattern curve.

2.6. Statistical analysis

The analysis of survival was carried out using the Kaplan–Meier test. The Log Rank test with a level of significance set at $P < 0.05$ was used for comparisons between groups.

The data from the immune parameters are expressed as the mean ± S.D. of the values from the number of surviving animals at each experimental time. Each value is the mean of the data from an assay performed in duplicate. The data were examined statistically by two-way analysis of variance (ANOVA). The Scheffe's F-test was used for the comparison of parametric samples. $P < 0.05$ was taken as the minimum significance level. The normality of the samples was tested by the Kolmogorov–Smirnov test.

3. Results

The survival times are shown in Fig. 1. The analysis of survival revealed statistically significant differences between the slow and the fast animals ($P < 0.05$), with the fast animals showing the higher survival time. Mean survival was 76 ± 5 and 89 ± 6 weeks for the slow and fast animals, respectively.

With respect to the tightrope test (Fig. 2), a higher percentage of fast animals than of slow animals was able to perform the test satisfactory. This neuromuscular capacity decreased with age in both groups, but more markedly in the slow mice. Moreover, the fast animals showed a neuromuscular coordination at 18 months of age similar to slow mice at the beginning of the experiment (6 months of age).

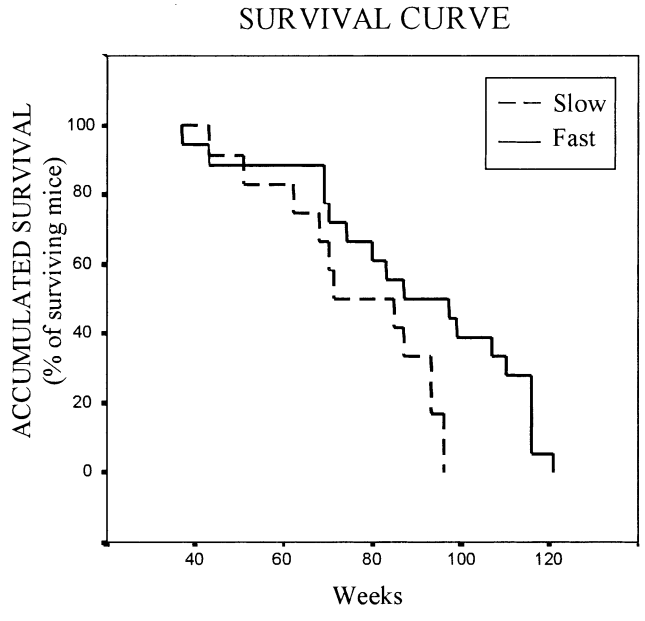

Fig. 1. Rate of survival of slow and fast female Swiss mice. Each line represents the accumulated survival of the animals. The fast group contained 18 animals and the slow group contained 12 animals at the start of the experiment. The survival mean of fast and slow mice is 89 ± 6 and 76 ± 5 weeks, respectively. The Log Rank statistics revealed significant differences between slow and fast mice ($P = 0.021$).

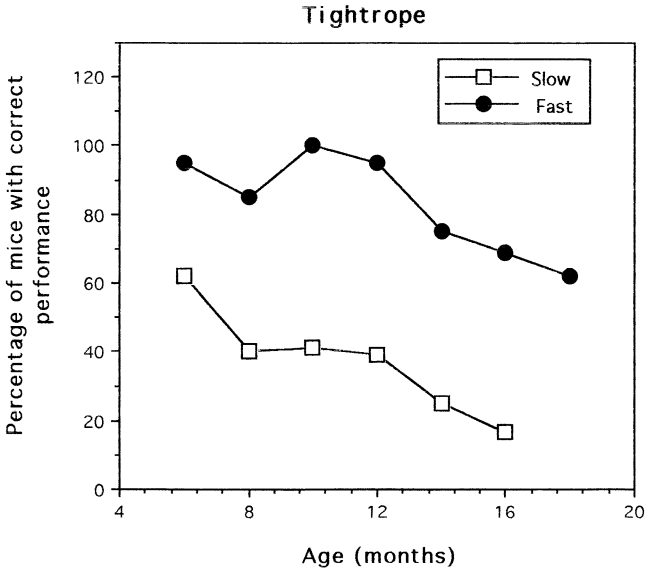

Fig. 2. Performance of female Swiss mice on a tightrope test of neuromuscular coordination. The line with white squares represents the slow mice and the line with dark circles represents the fast mice. Each point is the percentage of animals with correct performance from the number of surviving animals at each experimental time.

As regards the phagocytic process, it seemed better preserved in the fast than in the slow mice. The adherence indexes (AI) of the macrophages increased gradually with age ($P < 0.001$), in the fast group, from a value of 31 ± 4 at 6 months to 74 ± 4 at 17 months. The values from the fast mice were similar to those from the slow mice. The CI (Table 1) declined sharply

Table 1
Chemotaxis index of peritoneal macrophages from slow and fast female Swiss mice

Age (months)	Slow	Fast
6	681 ± 110	962 ± 121[c]
7	489 ± 112	710 ± 124[b]
8	470 ± 112	590 ± 98[a]
9	139 ± 38	474 ± 83[b]
10	212 ± 61	235 ± 59
11	88 ± 24	150 ± 34[a]
12	108 ± 33	161 ± 39
13	129 ± 42	175 ± 29
14	131 ± 21	181 ± 39
15	133 ± 35	218 ± 64[a]
16	166 ± 34	272 ± 64[a]
17	200 ± 36	327 ± 65[a]

Each date represents the mean ± S.D. of values from the number of surviving animals at each experimental time.

[a] $P < 0.05$.

[b] $P < 0.01$.

[c] $P < 0.001$ with respect to values of slow mice.

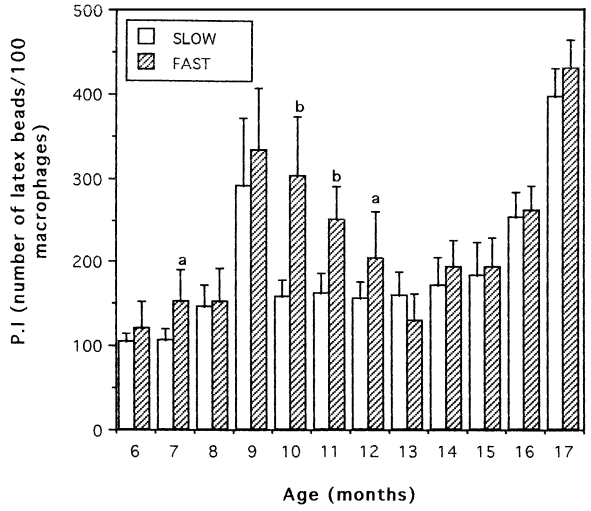

Fig. 3. Phagocytosis index (PI) of peritoneal macrophages from slow and fast female Swiss mice. Bars represent the mean ± S.D. of values from the number of surving animals at each experimental time. $^{a}P < 0.05$; $^{b}P < 0.01$ with respect to the values of slow mice.

until 10 months of life and then it began a slight rise. The statistical analysis showed a significant effect of age on this function ($P < 0.001$). The CI was higher in cells from the fast mice, with statistically significant differences between fast and slow mice at 6 ($P < 0.01$), 7 ($P < 0.01$), 8 ($P < 0.05$), 9 ($P < 0.01$), 15, 16 and 17 months ($P < 0.05$). The PI (Fig. 3) presented two peaks, one in the adult period and the other in old age. This index was higher in the fast mice with statistically significant differences between fast and slow mice at 7 ($P < 0.05$), 10, 11 ($P < 0.01$) and 12 months ($P < 0.05$). Intracellular anion superoxide production (Fig. 4) increased progressively with ageing ($P < 0.001$), with higher values in the macrophages from the fast mice in both stimulated and non-stimulated samples, and significant differences ($P < 0.05$ and < 0.001, respectively) between the fast and the slow animals. The extracellular production of superoxide (Fig. 5) increased with age ($P < 0.001$), but in contrast to the intracellular data, the extracellular production of this free radical was higher in the slow mice, increasing strikingly in the last months of life.

The evolution of the body weight with age is shown in Fig. 6. The two-way ANOVA analysis of the results revealed a significant main effect of the behavioural category ($P < 0.01$) and no effect with age. The fast mice were heavier than the slow mice, with statistical significance at 10, 12 and 14 months of age.

4. Discussion

In recent years, a considerable research effort has focused on the identification of biomarkers of ageing. Behavioural and immunologic tests have been proposed to assess physiological or functional age [23]. Accordingly we have proposed a model of premature mouse immunosenescence based on the performance in a simple T-maze test [9,14]. Moreover, we have previously demonstrated interindividual differences in the response of mice in identical behavioural tests, with the slow animals showing an increased level of emotionality/anxiety [43]. In the present work we have confirmed that a slow performance in the maze predicts a shorter life span, and is accompanied by a less competent immune system and neuromuscular capacity. Probably the slow mice suffer a premature impairment of the nervous–immune communication expressed in a higher age-related reactivity to stress. An efficient exploratory behaviour relies on an adequate level of CNS functions which support the ability of the fast mice to adapt quickly to novel stress-causing environments. Conversely, a less competent CNS would be responsible for the inadequate behaviour expressed in the high anxiety levels

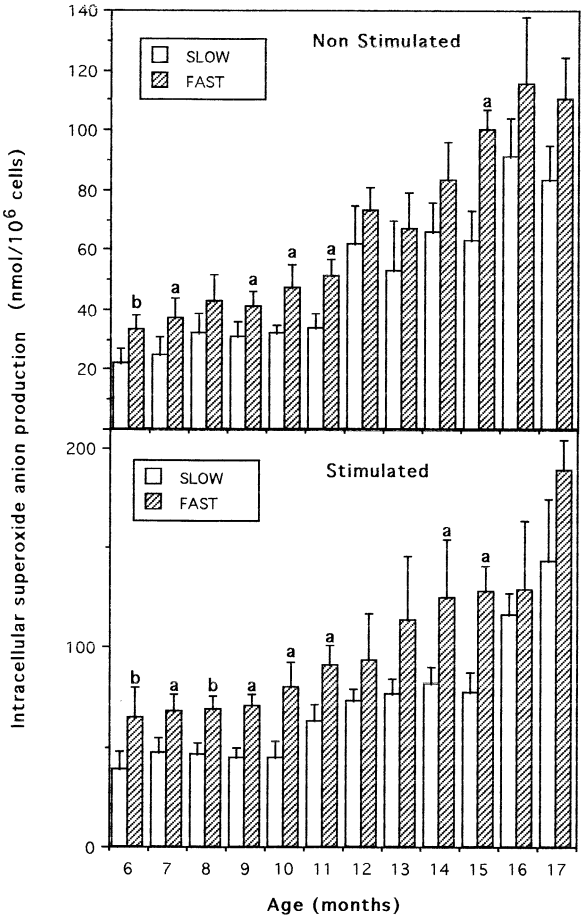

Fig. 4. Intracellular superoxide anion production (nmol/10^6 cell) in non-stimulated (upper figure) and stimulated samples (bottom figure) of peritoneal macrophages from slow and fast female Swiss mice. Bars represent the mean ± S.D. of values from the number of surviving animals at each experimental time. $^{a}P < 0.05$; $^{b}P < 0.01$ with respect to values of slow mice.

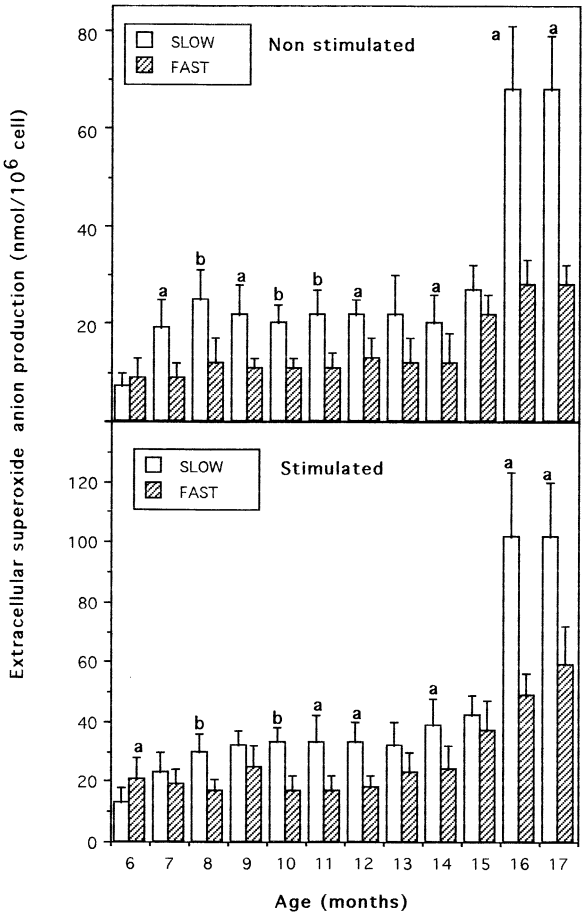

Fig. 5. Extracellular superoxide anion production (nmol/10^6 cell) in non-stimulated (upper figure) and stimulated samples (bottom figure) of peritoneal macrophages from slow and fast female Swiss mice. Bars represent the mean ± S.D. of values from the number of surviving animals at each experimental time. $^{a}P < 0.05$; $^{b}P < 0.01$ with respect to values of slow mice.

and concomitant slow exploration or immobility (freezing behaviour) of the slow mice.

These findings confirm the presence of a consistent relationship between performance in the T-maze and immunosenescence, as previously suggested by De la Fuente et al. [14], and Correa et al. [9]. The physiological basis of this relationship might be related to neural connections in the limbic region (hippocampus, amygdala) and the cerebral cortex, which play a key role in the integrated CNS–immune reactions to psychological stressful factors that exert an influence on the immune response [1,27]. Current research suggests that the behavioural impairment with age is the result of a dysfunction in the neurotransmission but not a loss of neurons of the CNS [34,35]. This is in agreement with our latest findings suggesting that fast and slow mice differ at the neurochemical level in several areas of the CNS. Nevertheless, some authors [41] have suggested that synaptic loss is a consequence of a disease process but not of normal ageing.

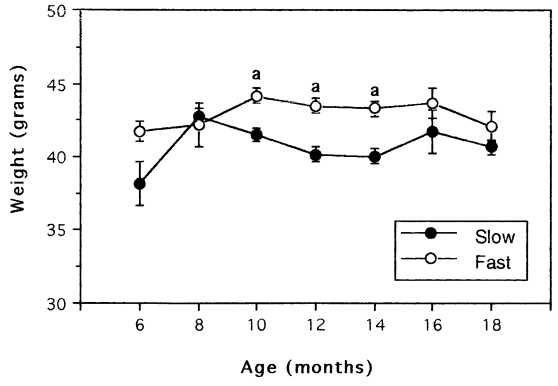

Fig. 6. Body weight (grams) of ageing slow and fast female Swiss mice. The values represent the mean ± S.D. of data obtained from the number of surviving animals. $^{a}P < 0.05$ with respect to values of slow mice.

As we have mentioned above, other authors have pointed out that hyper-reactivity to stressors in mice and rats is genetically linked to a shorter life span and to an acceleration of neurodegenerative changes in the brain [2,10]. Moreover, it has been suggested that environmental influences may result in an impaired physiological adaptation to stress and an acceleration of the ageing process, with higher mortality. Therefore, Gilad and Gilad [19] conclude that there is some evidence in favour of a 'stress–longevity–neurodegeneration' hypothesis of ageing.

On the other hand, a slow performance in the T-maze is accompanied by a lower ability to perform in the tightrope test as it was demonstrated by our previous cross-sectional research [22]. Accordingly, in this longitudinal study this capacity was lower in the slow mice as compared to the fast mice, with the fast mice at the end of the experiment showing the same neuromuscular capacity than the slow mice at the beginning, which indicates than the slow mice, with respect to the neuromuscular coordination and vigor, are functionally impaired. This impaired ability of the slow mice in the tightrope test is not linked to an increase in the body weight of those animals since the fast mice were heavier than the slow mice. In agreement with other authors [25], since the neuromuscular capacity showed a correlation with life span, it could be considered that the slow mice have a higher biological age than the fast mice. Therefore, this parameter could be a useful biomarker of ageing.

Regarding the immune system, while most investigations of age-related changes in this system have focused mainly on lymphocyte function, which is decreased [28,38], less attention has been paid to phagocytes. Thus, the study of these cells has provided few and contradictory data [11,31,37]. The present longitudinal study might throw some light on this controversy, since depending on the mouse age we can see an increment, a decrement or no change in phagocytic functions. The

high indexes of chemotaxis (a crucial function involved in immune and inflammatory responses) found in the fast animals indicate that the macrophages of these animals have a better migration, which is essential for immune surveillance and for promoting cell–cell interactions involved in the immune response [7]. This fast group also shows a better capacity of foreign material ingestion by macrophages, which is required for the defense of the organism against pathogenic agents [36]. It is specially interesting that the macrophages from fast mice show a higher production of intracellular superoxide, which enables foreign material destruction and high bacteria-killing activity. However, the slow mice show the higher levels of extracellular superoxide, which reflects a harmful imbalance in the production of this anion, since its presence in the extracellular medium can lead to serious host tissue damage. Since ageing is linked to an increased rate of free radical generation and a decline in antioxidant competence [31,40] the age-related alterations of the immune functions probably result from oxidative stress [32].

As pointed out by Wayne et al. [44], and Aspinall [3], the immunity of an animal is related not only to its health but to its survival as well. Accordingly, our present data show that age-dependent changes in immune parameters are related to survival. In fact, in our opinion the most likely cause of early death in the slow mice would be a premature appearance of the tumours which, according to the suppliers of our mice and to our own observations, are the most frequent cause of death in this mouse strain. Thus, an impaired immune function would render the animals more tumour-prone, in agreement with the well known relationship between immune deterioration and tumourigenesis [28,29].

In agreement with the above, it could be considered that the slow mice are 'biologically older' than the fast mice with regard to functional mechanisms implicated in exploratory behaviour, neuromuscular vigor and immune competence. The hypothesis of an accelerated ageing of the slow mice is in agreement with their shorter life span shown in Fig. 1. These and previous data [42] confirm that the T-maze methods provide a simple and fast approach for determination of murine biological age that can be useful for preliminary studies prior to the application of more comprehensive batteries of tests [21,26,30].

Summing up, our data show that slow mice suffer an early immune decline and CNS impairment (expressed in a worse behavioural response in a T-maze) and a shortening of life span, thus providing new evidence on the close relationship between the immune and the nervous system and their central role in the physiopathology of ageing and determination of life span. Since, slow mice are characterised by early age-related immune system changes accompanied by an increased emotionality/anxiety, this murine model of premature ageing may be useful for studies on 'biological age' and pharmacological and nutritional life extension in mice. Further physiological and neurochemical research as well as the analysis of additional immunological parameters may provide stronger backing for this view.

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