ELSEVIER

Experimental Gerontology 37 (2002) 249–256

Experimental Gerontology

www.elsevier.com/locate/expgero

Leukocyte function and life span in a murine model of premature immunosenescence

N. Guayerbas[a], M. Puerto[a], V.M. Víctor[a], J. Miquel[b], M. De la Fuente[a,*]

[a]*Department of Animal Physiology, Faculty of Biological Sciences, Complutense University, 28040 Madrid, Spain*
[b]*Department of Pharmacology, University of Alicante, Alicante, Spain*

Received 1 June 2001; received in revised form 20 September 2001; accepted 26 September 2001

Abstract

Aging associates with a decline of physiological functions, including the function of the nervous and the immune system. These aged-related changes occur in various degrees in different members of a mouse outbred population. Accordingly, we have proposed a model of premature immunosenescence in mice, based on the demonstration of premature decline in the behavioral response in a simple T-maze and in several immune functions in Swis outbred mice. Those mice with a worst (slow) performance in this test (linked to a higher emotional response to stress) show a shorter life span and a decreased immune function when compared to fast mice. In order to provide biomarkers of 'biological aging' related to health and survival, the present longitudinal study includes the analysis of several immunological parameters such as, proliferative response to mitogen Con A, NK activity and cytokine (TNFα, IL-1β and IL-2) release by peritoneal leukocytes from female Swiss mice. Slow mice showed a lower proliferative response to Con A, IL-2 and IL-1β release, an impaired NK activity and an increased TNFα production as compared to fast mice. Moreover, the age-associated decline of these functions is more strikingly slow than in fast mice. In summary, we propose the above immunological parameters, that change with aging at a different rate in members of a same population, as useful biomarkers to asses the rate of biological aging in mice. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Leukocyte function; Immunosenescence model; Life span; Mice

1. Introduction

Aging is associated with a decline of many physiological functions, including those of the nervous (Rapp et al., 1999; Carrié et al., 1999) and the immune system (McArthur, 1998; Solana and Pawlec, 1998; Medina et al., 2000), as well as in their bidirectional communication (Fabris, 1991; Goya and Bolognani, 1999), which leads to a loss of homeostasis enhancing the probability of death. Thus, longevity has been related with nervous system function, as assessed by behavioral response (Ingram and Reynolds, 1986), and with immune competence (Wayne et al., 1990; Ginaldi et al., 1999).

Cumulative evidences suggest an association between response to stressful stimuli and longevity (Sandi et al., 1992; Kvist, 1993). In particular, animals which exhibit immobility or 'freezing behavior' when placed in a new environment usually show a short life (Dellu et al., 1994). Several authors have pointed out the crucial importance of the nervous–immune

* Corresponding author. Tel.: +34-91-3944989; fax: +34-91-3944935.
E-mail address: mondelaf@eucmax.sim.ucm.es (M. De la Fuente).

0531-5565/02/$ - see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S0531-5565(01)00190-5

network in the adaptation to environmental stimuli in order to maintain homeostasis (Stein-Behrens and Sapolsky, 1992; Tomaszewska and Przekop, 1997). In the elderly a dysregulation of the nervous–immune relation is accompanied by an impaired stress response contributing to morbidity and mortality during senescence (Wilder, 1995; Dhar, 1998).

Recent studies carried out in our laboratory have shown that interindividual differences in the behavioral response in a simple T-maze test are related to life span among members of a Swiss outbred mouse population. Mice which quickly explore the maze (fast mice) reach a longer life span than those, which show a slow performance in the T-maze (Guayerbas et al., 2000). Moreover, the slow mice show a lower neuromuscular coordination and vigor in comparison to the fast mice, as assessed by a tight-rope test (Miquel and Blasco, 1978) similar to that used by Ingram (1983) for behavioral determination of biological age in the laboratory mouse. The slow mice also show, when compared to fast mice, a decreased locomotor activity and an increased level of emotionality/anxiety as detected by three standard behavioral tests (Viveros et al., 2001). In addition, previous cross-sectional studies revealed that several functions of leukocytes, which decrease with age, are more impaired in the slow mice (De la Fuente et al., 1998; Correa et al., 1999; Viveros et al., 2001). In view of the fact that ageing results in 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.

The present longitudinal study offers further evidence of the usefulness of determination of immunological parameters to provide biomarkers of 'biological age', related to health and survival.

2. Materials and methods

2.1. Animals

We have used female OF1 Swiss mice (*Mus musculus*) (Harlan Ibérica, Barcelona, Spain), which were 15 week old on arrival to our laboratory. The mice were specific pathogen free, as tested by Harlan according to FELASA recommendations. They were randomly divided in groups of 5, 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 behavior 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, 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 14 mice contained the fast population and the other, of 8 mice, the slow population with a fast/slow mouse ratio of 100/0 and 0/100, respectively. This test was performed always between 8:00 and 10:00 h, under red light.

2.3. Collection of peritoneal leukocytes

In this longitudinal study, the peritoneal suspensions were obtained at 24, 32, 36, 45, 68 and 80 weeks of age between 8:00 and 10:00 h, without sacrificing the mice.

Mice were held by the cervical skin, the abdomen was cleansed with 70% ethanol and 3 ml of sterile

Hank's solution injected intraperitoneally. After massaging the abdomen, 80% of the injected volume was recovered. Peritoneal leukocytes were counted in Neubauer chambers (Blau Brand, Germany) and adjusted to 1×10^6 cells/ml. The cellular suspensions showed a viability of $99 \pm 1\%$. The cells were resuspended in RPMI 1640 enriched with L-glutamine (Gibco, Canada Ltd, Burlington, Ont.) and supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS) (Gibco) and gentamicin (100 mg/ml, Gibco).

2.4. Proliferation assay

A previously described method was used (Del Río et al., 1994). Aliquots of 200 μl were dispensed in plates of 96 wells (Orange Scientific, Belgium) and 20 μl of Concanavaline A (Con A, 1 μg/ml, Sigma, St Luois, MO) were added to each well. Plates were incubated for 48 h. Then 0.5 μCi ^{3}H-thymidine were added to each well and after 8 h the cells were harvested in a semiautomatic microharvester and thymidine uptake was measured in a beta counter (LKB) for 1 min. The results were expressed as ^{3}H-thymidine uptake (cpm).

2.5. Cytotoxicity assay

An enzimatic colorimetric assay was used for cytolysis measurements of target cells (Cytotox 96 TM Promega, Boeringher Ingelheim) based on the determination of LDH using tetrazolium salts, as previously used by us on this kind of samples (Ferrández et al., 1999). Murine lymphoma YAC-1 cells were used as target in the NK assay. The cells were maintained in complete medium (RPMI-1640 plus 10% fetal calf serum, Life Technologies). Target cells were seeded in 96-well U bottom culture plates (Orange Scientific, Belgium) at 10^4 cells/well in 1640 RPMI without phenol red. Effector cells, i.e. peritoneal leucocytes, were added at 10^5 cells/well, being the effect/target rate of 10/1. The plates were centrifuged at $250 \times g$ for 4 min to facilitate cell contacts and then they were incubated for 4 h at 37°C. After incubation, lactate dehydrogenase enzymatic activity was measured in 50 μl/well of the supernatants by addition of the enzyme substrate and absorbance recording at 490 nm. Three kinds of control measurements were performed: a target spontaneous release, a target maximum release, and an effector spontaneous release. To determine the percentage of lysis of target cells, the following equation was used: $\%\ \text{lysis} = ((E - \text{ES} - \text{TS})/(M - \text{ES} - \text{TS})) \times 100$, where E is the mean of absorbances in the presence of effector cells; ES, the mean of absorbances of effector cells incubated alone; TS, the mean of absorbances in target cells incubated with medium alone; and M is the mean of maximum absorbances after incubating target cells with lysis solution.

2.6. TNFα and IL-1β production assay

Mouse tumor necrosis factor (TNFα) and interleukin-1β (IL-1β) productions were measured in the supernatants of cultures of peritoneal macrophages. Cell suspensions were incubated following the method previously described (Víctor et al., 2000) with Hank's solution at a final concentration of 2×10^5 cells/200 μl/well in 96 wells plates for 60 min to allow macrophage to form a monolayer, and after this time lypopolysaccharide (LPS, *E. coli*, 055:B5, Sigma, 10 μg/ml) was added. After 24 h of incubation the supernatants were collected and the concentration of cytokines was measured using an ELISA kit (Endogen, Woburn, USA) and the results were expressed as pg/ml.

2.7. IL-2 production assay

The concentration of interleukin-2 (IL-2) was determined on culture supernatants of peritoneal leukocytes following a method previously described by us (Medina et al., 2000). After 48 h of incubation with the mitogen Con A (Sigma, 1 μg/ml) the supernatants were collected and measured using an ELISA kit (R and D System, Minneapolis, USA) and the results were expressed as pg/ml.

2.8. Statistical analysis

The data are expressed as the mean ± S.D. The normality of the samples was confirmed by the Kolmogorov–Smirnov test and the homogeneity of variances by the Levene test. The two-way analysis of variance (ANOVA) and the Tukey test were used for the comparison of parametric samples.

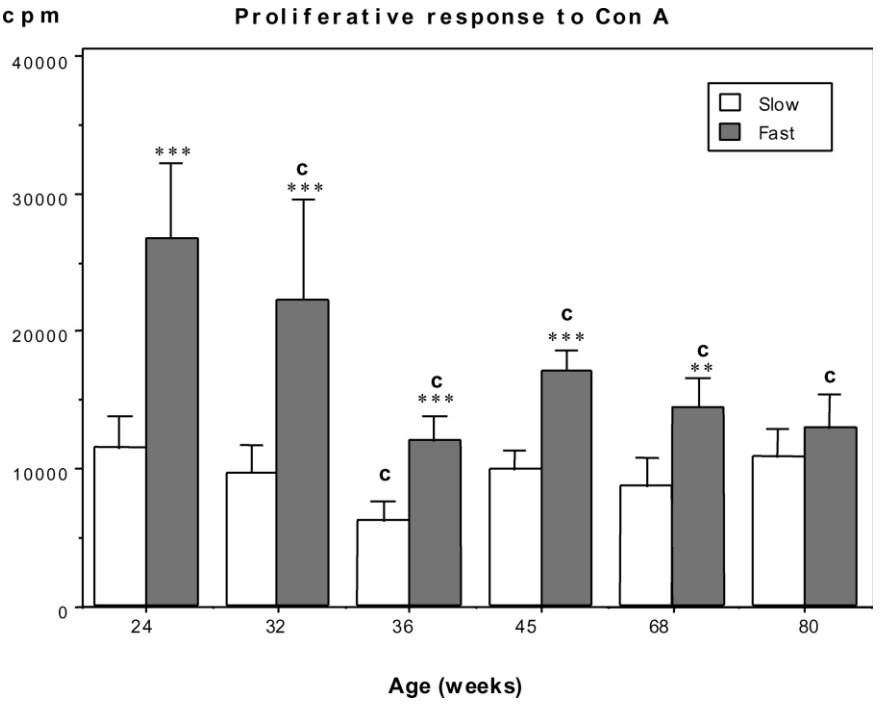

Fig. 1. Proliferative response to Con A (1 μg/ml) of peritoneal leukocyte of slow and fast Swiss mice. The data represent the mean ± S.D. of values from the number of surviving animals at each experimental time. $^{**}p < 0.01$; $^{***}p < 0.001$ with respect to the values from the slow mice. $p < 0.001$ with respect to values of the adults (24 weeks).

3. Results

Results of the proliferative response to Con A of peritoneal leukocytes are shown in Fig. 1 The two-way ANOVA analysis revealed statistically significant differences between both groups, i.e. slow and fast mice ($p < 0.001$), with the fast mice showing the highest value. The analysis of the data showed a decrease in IL-2 production with age in both groups ($p < 0.001$). Fig. 2 shows the data of IL-2 production. A significant effect of age was found for this function ($p < 0.001$). The comparisons of slow and fast mice revealed significant differences at 24 ($p < 0.05$) and 32 ($p < 0.05$) weeks of life, whereas at the other ages studied there were only trends suggesting higher values for the fast than for the slow mice.

Table 1 shows the data from NK activity, IL-1β and TNFα production of peritoneal leukocytes and the survival of the animals. NK cell activity did not show significant differences between fast and slow mice except at 68 ($p < 0.001$) and 80 ($p = 0.033$) weeks of age, when the fast mice showed higher values than the slow mice. This function showed significant changes with age in both, fast and slow mice, ($p < 0.01$). TNFα production by peritoneal leukocytes was significantly higher in the slow than in the fast mice ($p = 0.01$) and experimented an increase with age ($p < 0.001$). Analysis of the data on IL-1β production revealed significant differences between both groups of mice ($p = 0.028$), with the fast mice showing the highest value. Two-way ANOVA analysis showed a significant effect of age ($p < 0.001$) in this activity.

Finally, survival analysis using the Kaplan–Meier test revealed significant differences between slow and fast mice ($p = 0.028$). Whereas the mean survival of the slow mice was 86.3, fast mice survival was 97.5 weeks. Furthermore, slow mice reached 96 weeks of life (highest survival) and the fast mice reached 121 weeks.

4. Discussions

Research on aging has revealed that ‘biological

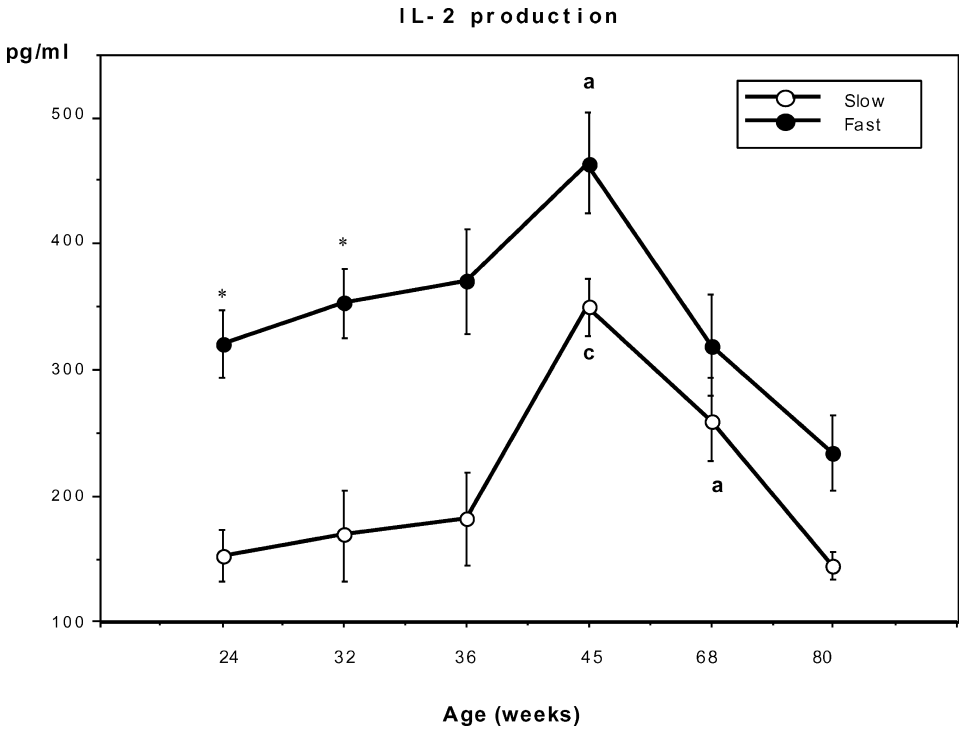

Fig. 2. IL-2 release in response to Con A (μg/ml) of peritoneal leukocyte of slow and fast Swiss mice. The data represent the mean ± SD of values from the number of surviving animals at each experimental time. $*p < 0.05$ with respect to the values from the slow mice. $p < 0.05$; $p < 0.001$ with respect to values of the adults (24 weeks).

Table 1
Peritoneal leukocyte functions and survival of slow and fast Swiss mice (The data represent the mean ± SD of values from the number of surviving animals at each experimental time. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ with respect to the values from the slow mice. $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$; $^{\dagger\dagger\dagger}p < 0.001$ with respect to values of the adults (24 weeks). The survival values are expressed as percentage of surviving mice (% accumulated survival))

Age	24	32	36	45	68	80
Functions						
NK (% lysis)						
Slow	68 ± 5	64 ± 6	47 ± 13††	42 ± 7††	45 ± 4†	40 ± 13††
Fast	70 ± 6	63 ± 15	39 ± 10†††	41 ± 5†††	63 ± 2***	55 ± 4**
TNFα (pg/ml)						
Slow	47 ± 10	64 ± 8†††	95 ± 9†††	108 ± 22†††	100 ± 25†††	98 ± 13†††
Fast	34 ± 10**	74 ± 17†††	82 ± 8†††	85 ± 15†††	86 ± 10†††	90 ± 5†††
IL-1β (pg/ml)						
Slow	229 ± 49	203 ± 26	126 ± 27†††	170 ± 1†	116 ± 15†††	104 ± 3†††
Fast	214 ± 51	208 ± 24	295 ± 14*†	397 ± 52*†††	301 ± 29***†	206 ± 33*
Survival (% accumulated survival)						
Weeks	70	80	90	100	110	120
Slow	75	75	50	0	0	0
Fast	75	67	67	50	42	8

aging' is not equivalent to 'chronological aging' (Collier and Coleman, 1991; Gallagher and Rapp, 1997), i.e. that age-related changes in physiological systems, and in behavioral competence occur to varying degrees in different members of a population. Therefore, there is growing interest in the identification of biomarkers of aging using mouse models to assess the effects of age (Ingram and Jucker, 1999). Accordingly, we have proposed a model of premature mouse immunosenescence associated with a worst (slow) performance in a simple T-maze (De la Fuente et al., 1998; Correa et al., 1999). Moreover, in a recent work (Viveros et al., 2001) we have provided a complete behavioral characterization of slow and fast animals, showing a higher emotional response to stress accompanied by a reduced mobility or 'freezing' in the slow mice. Thus, we have demonstrated a shorter life span (Guayerbas et al., 2000) and a worst phagocytic function (Correa et al., 1999) of the slow mice as compared to the fast mice.

It is now well established that the nervous and the immune system are involved in a bidirectional communication (Elenkov et al., 2000) that is very important for the maintenance of physiological homeostasis in response to stressors (Haas and Schauenstein, 1997), and therefore in biological ageing (Fabris, 1991). Results suggest that slow mice suffer a premature impairment of the nervous–immune communication manifested by an increased reactivity to stress, which correlates with a shortening of life span. This is in agreement with other authors who pointed out that inherent hyper-reactivity to stressors is genetically linked to a shorter life span and to accelerate age-dependent neurodegenerative changes in the brain (Gilad and Gilad, 1995).

Results accumulated in the last years indicate a decline of the immune system with age (McArthur, 1998; Miyaji et al., 2000; Ortega et al., 2000). In relation, the effect of age on several functions of lymphocytes, different investigators have observed a significantly decreased lymphocyte proliferation (for review see Chakravarti and Abraham, 1999; Pawelec, 1999) as well as an impaired synthesis and response to IL-2 (Pahlavani and Richardson, 1996; Medina et al., 2000) which hamper the proper functioning of the lymphocytes. The activity and cytotoxic capacity of NK cells during aging have been studied extensively, and contrasting results have been published. Most research reported a decrease with aging (Albright and Albright, 1998; Ferrández et al., 1999; De la Fuente et al., 2001). Aging also causes many changes in macrophage functions, including an altered ability for the production of different cytokines such as IL-1β, IL-6 and TNFα (Fagiolo et al., 1993; Wallace et al., 1995) molecules that play an important regulatory role in T-cell activation (Ceuppens et al., 1988).

In the present work, we have analyzed these functions in fast and slow animals in a longitudinal experiment. We have demonstrated the premature immunosenescence of slow mice, shown by a reduced lymphoproliferation and IL-2 release when compared with fast mice. An immune-response pattern of poor T-cell proliferation and low IL-2 production was found to be predictive of higher mortality in the elderly (Ferguson et al., 1995). Whereas a substantial decline in NK activity is found in the slow mice, the fast mice show a tendency to recover this activity in old age. This could be beneficial for the prolongation of life span, since there is an increment of abnormal self-cells with advancing aging. These results are in agreement with those reported by Miyaji et al. (2000). Moreover, it has been suggested that a persistenly low NK activity is a predictor of impending morbidity (Levy et al., 1991). Regarding macrophage cytokine release, our results show that TNFα increases with aging in both slow and fast mice, in agreement with the literature (Fagiolo et al., 1993; Rink et al., 1998), although the former shows the highest value. An increased production of proinflamatory cytokines, such as TNFα, could explain many aspects of the age-associated pathological events (Frasca et al., 1997). IL-1β shows higher values in fast than in slow mice. Although IL-1β is a proinflamatory cytokine it shows differences with the TNFα, since it is needed to stimulate T lymphocytes in the antigenic presentation and plays an important role in many biological responses to infection.

Considering the relevance of an optimal immune function for successful aging, we propose the above immune parameters as useful biomarkers for assessment of 'biological age' and predictors of morbidity and longevity. In fact, slow mice, which show values more similar to those of older animals for the above immune parameters, have a shorter lifespan than the fast animals. It is also of interest to note the usefulness of this prematurely aged mouse model

of immunosenescence to study the effect of age and to test pharmacological and nutritional treatments designed to improve immune function in the elderly.

Acknowledgements

The authors wish to acknowledge the support of the present work by FISS Grants (97/2078 and 99/1264) and CAM Grant (08.5/0015/1997).

References

Albright, J.W., Albright, J.F., 1998. Impaired natural killer cell function as a consequence of aging. Exp. Gerontol. 33, 13–25.

Carrié, I., Debray, M., Bourre, J.M., Francès, H., 1999. Age-induced cognitive alterations in OF1 mice. Physiol. Behav. 66, 651–656.

Ceuppens, J.L., Baroja, M.L., Lorre, K., van Damme, J.V., Billiau, A., 1988. Human T cell activation with phytohemagglutinin. The function of IL-6 as an accessory signal. J. Immunol. 141, 3868–3874.

Chakravarti, B., Abraham, G.N., 1999. Aging and T-cell mediated immunity. Mech. Ageing Dev..

Collier, T.J., Coleman, P.D., 1991. Divergence of biological and chronological aging: evidence from rodent studies. Neurobiol. Aging 12, 685–693.

Correa, R., Blanco, B., Del Río, M., Víctor, V.M., Guayerbas, N., Medina, S., De la Fuente, M., 1999. Effect of a diet supplemented with thioproline on murine macrophage function in a model of premature aging. Biofactors 10, 195–200.

De la Fuente, M., Miñano, M., Víctor, V.M., Del Río, M., Ferrandez, M.D., Díez, A., Miquel, J., 1998. Relation between exploratory activity and immune function in aged mice. Mech. Ageing Dev. 102, 263–277.

De la Fuente, M., Del Río, M., Víctor, V.M., Medina, S., 2001. Neuropeptide Y effects on murine natural killer activity: changes with ageing and cAMP involvement. Regul. Pept. 101, 73–79.

Del Río, M., Hernánz, A., De la Fuente, M., 1994. Bombesin, gastrin-releasing peptide and neuromedin C modulate murine lymphocyte proliferation through adherent accessory cells and activate protein kinase C. Peptides 15, 15–22.

Dellu, F., Mayo, W., Vallée, M., Le Moal, M., Simon, H., 1994. Reactivity to novelty during youth as a predictive factor of cognitive impairment in the elderly: a longitudinal study in rats. Brain Res. 653, 51–56.

Dhar, H.L., 1998. Physiology of aging. Indian J. Med. Sci. 52, 485–497.

Elenkov, I.J., Wilder, R.L., Chrousos, G.P., Vizi E.S., 2000. The sympathetic nerve-an integrative interface between two supersystems: the brain and the immune system. Pharmacol. Rev. 52, 595–638.

Fabris, N., 1991. Neuroendocrine–immune interactions: a theoretical approach to aging. Arch. Gerontol. Geriatr. 12, 219–230.

Fagiolo, U., Cossarizza, A., Scala, E., Fanales-Balasio, E., Ortolani, C., Cozzi, E., Monti, D., Franceschi, C., Paganelli, R., 1993. Increased cytokine production in mononuclear cells of healthy elderly people. Eur. J. Immunol. 23, 2375–2378.

Ferguson, F.G., Wikby, A., Maxson, P., Olsson, J., Johansson, B., 1995. Immune parameters in a longitudinal study of a very old population of Swedish people: a comparison between survivors and nonsurvivors. J. Gerontol. 50, 378–382.

Ferrández, M.D., Correa, R., Del Río, M., De la Fuente, M., 1999. Effects in vitro of several antioxidants on the natural killer function on aging mice. Exp. Gerontol. 34, 675–685.

Frasca, D., Pucci, S., Goso, C., Barattini, P., Barile, S., Pioli, C., Doria, G., 1997. Regulation of cytokine production in aging: use of recombinant cytokynes to up-regulate mitogen stimulated spleen cells. Mech. Ageing Dev. 93, 157–169.

Gallagher, M., Rapp, P.R., 1997. The use of animal models to study the effects of aging on cognition. Annu. Rev. Psychol. 48, 339–370.

Gilad, G.M., Gilad, V.H., 1995. Strain, stress, neurodegeneration and longevity. Mech. Ageing Dev. 78, 75–83.

Ginaldi, L., De Martinis, M., Dòstilio, A., Marini, L., Loreto, M.F., Quaglino, D., 1999. Immunological changes in the elderly. Aging Clin. Exp. Res. 11, 281–286.

Goya, R.G., Bolognani, F., 1999. Homeostasis, thymic hormones and aging. Gerontology 45, 174–178.

Guayerbas, N., Sánchez, A.I., Gamallo, A., Miquel, J., De la Fuente, M., 2000. Mouse performance in an exploratory activity test as a longevity biomarker. In: Tur-Mar, J.A., Orellaua-Muriana, J.M. (Eds.), Animal Research and Welfare – A Partnership. Laboratory Animals Ltd., London, pp. 159–161.

Haas, H.S., Schauenstein, K., 1997. Neuroimmunomodulation via limbic structures-the neuroanatomy of psychoimmunologyu. Prog. Neurobiol. 51, 195–222.

Ingram, D.K., 1983. Toward the behavioral assessment of biological aging in the laboratory mouse: concepts, terminology, and objectives. Exp. Aging Res. 9, 225–238.

Ingram, D.K., Jucker, M., 1999. Developing mouse models of aging: a consideration of strain differences in age related behavioral and neural parameters. Neurobiol. Aging 20, 137–145.

Ingram, D.K., Reynolds, M.A., 1986. Assessing the predictive validity of psychomotor tests as measures of biological age in mice. Exp. Aging Res. 12, 155–162.

Kvist, S.B.M., 1993. Impact of age and situation-specific experience on maze learning-sensitive open-field parameters in mice. Exp. Aging Res. 19, 259–275.

Levy, S.M., Herberman, R.B., Lee, J., Whiteside, T., Bedle, M., Heiden, L., Simons, A., 1991. Persistently lo natural killer cell activity, age, and environmental stress as predictors of infectious moribidity. Nature Immun. Cell Growth Regul. 10, 536–539.

McArthur, W.P., 1998. Effect of aging on immunocompetent and inflamatory cells. Periodontology 2000 16, 53–79.

Medina, S., Del Río, M., Hernanz, A., De la Fuente, M., 2000. Age-related changes in the neuropeptide Y effects on murine lymphoproliferation and interleukin-2 production. Peptides 21, 1403–1409.

Miquel, J., Blasco, M., 1978. A simple technique for evaluation of vitality loss in aging mice, by testing their muscular coordination and vigor. Exp. Gerontol. 13, 389–396.

Miyaji, C., Watanabe, H., Toma, H., Akisaka, M., Tomiyama, D., Sato, Y., Abo, T., 2000. Functional alteration of granulocytes, NK cells, and natural killer T cells in centenarians. Human Immunol. 61, 908–916.

Ortega, E., García, J.J., De la Fuente, M., 2000. Ageing modultates some aspects of the non-specific immune response of murine macrophages and lymphocytes. Exp. Physiol. 85 (55.5), 519–525.

Pahlavani, M.A., Richardson, A., 1996. The effect of age on the expression of interleukin-2. Mech. Ageing Dev. 89, 125–154.

Pawelec, G., 1999. Immunosenescence: impact in the young as well as the old?. Mech. Ageing Dev. 108, 1–7.

Rapp, P.R., Stack, E.C., Gallagher, M., 1999. Morphometric studies of the aged hippocampus: I. Volumetric analysis in behaviorally characterized rats. J. Comp. Neurol. 403, 459–470.

Rink, L., Cakman, I., Kirchner, H., 1998. Altered cytokine production in the elderly. Mech Ageing Dev. 102, 199–209.

Sandi, C., Borrell, J., Guaza, C., 1992. Behavioral factors in stress-induced immunomodulation. Behav. Brain Res. 48, 95–98.

Solana, R., Pawlec, G., 1998. Molecular and cellular basis of immunosenescence. Mech. Ageing Dev. 102, 115–129.

Stein-Behrens, B.A., Sapolsky, R.M., 1992. Stress, glucocorticoids, and aging. Aging Clin. Exp. Res. 4, 197–210.

Tomaszewska, D., Przekop, F., 1997. The immune–neuroendocrine interactions. J. Physiol. Pharmacol. 48, 139–158.

Víctor, V.M., Guayerbas, N., Puerto, M., Medina, S., De la Fuente, M., 2000. Ascorbic acid modulates in vitro the function of macrophages from mice with endotoxic shock. Immunopharmacology 46, 89–101.

Viveros, M.P., Fernández, B., Guayerbas, N., De la Fuente, M., 2001. Behavioral characterization of a mouse model of premature immunosenescence. J. Neuroimmunol. 114, 80–88.

Wallace, P.K., Eisenstein, T.K., Meissler, J.J., Morhan, P.S., 1995. Decreases in macrophage mediated antitumor activity with aging. Mech. Ageing Dev. 33, 313–322.

Wayne, S.J., Rhyne, R.L., Garry, P.J., Goodwin, J.S., 1990. Cell-mediated immunity as a predictor of morbidity and mortality in subjects over 60. J. Gerontol. 45, M45–M48.

Wilder, R.L., 1995. Neuroendocrine–immune system interactions and autoimmunity. Annu. Rev. Immunol. 13, 307–338.