

## Effects of social stress on tumor development in dominant male mice with diverse behavioral activity profiles

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We examined the influence of individual psychological profile and social behavior on tumor development in dominant male mice. Male OF1 mice were subjected to an open field test (OFT) to observe their motor activity and latency. Subsequently, the animals were divided into three groups: Stress-Non-Inoculated (SNI), Stress-Inoculated (SI) and Control-Inoculated (CI). The SI and CI groups were inoculated with tumor cells and the SNI group with vehicle. SNI and SI were exposed to social stress with an anosmic intruder six (T1) and twenty one (T2) days after inoculation and their behavior was analyzed. After T2, subjects were put down and the pulmonary metastatic foci counted. SI developed greater pulmonary metastasis than CI, indicating an effect of stress despite the animal's dominant status. Active animals developed less pulmonary metastasis than their passive counterparts. No differences were found in social behavior at T1. Differences were found, however, in some behavioral categories at T2 between SI and SNI, and between active and passive animals. These differences indicate an effect of tumor development on social behavior that is more evident in passive subjects.

*Efectos del estrés social en el desarrollo tumoral de ratones macho dominantes con diferentes perfiles de actividad conductual.* Se examinó la influencia del perfil psicológico individual y del comportamiento social en el desarrollo tumoral de ratones macho dominantes. Los animales fueron sometidos a un test de campo abierto (OFT) para medir la actividad locomotora y la latencia. Posteriormente, los animales se dividieron en tres grupos: Stress-No-Inoculado (SNI), Stress-Inoculado (SI) y Control-Inoculado (CI). SI y CI fueron inoculados con células tumorales y SNI con vehículo. Los grupos SI y CI fueron sometidos a estrés mediante la interacción con un animal intruso anósmico, seis (T1) y veintidós (T2) días después de la inoculación y analizada su conducta. Finalmente los animales fueron sacrificados y se contaron las metástasis pulmonares. SI desarrolló más metástasis que CI, indicando un efecto del estrés a pesar de su estatus de ganador. Los animales activos desarrollaron menos metástasis que los pasivos. Aunque no se encontraron diferencias conductuales a T1, sí se encontraron diferencias a T2 entre SI y SNI, y entre activos y pasivos. Estas diferencias indican que existe un efecto del desarrollo tumoral en la conducta social que es más evidente en los sujetos pasivos.

Diverse studies carried out in animals and humans have demonstrated that stress may modify the immune activity, making the host more susceptible to the development of neoplastic processes (Ben-Eliyahu, Yirmiya, Liebeskind, Taylor, & Gale, 1991; Kiecolt-Glaser & Glaser, 1999; Moynihan & Ader, 1996; Thomas, Pandey, Ramdas, & Nair, 2002). One method of studying this question has been to use animal models of social stress, which due to their ethological validity, may help clarify such phenomena in humans (Azpíroz, Garmendia, Fano, & Sánchez-Martín, 2003; Bartolomucci et al., 2001; Stefanski, 1998, 2000; Stefanski & Ben-Eliyahu, 1996). One of the most interesting aspects of the study of the impact of social stress on the immune activity and tumor development is the importance of the individual

characteristics expressed by subjects in these situations (i.e. coping strategies). Thus, for example, in situations not involving social interaction, an association has been found between individual differences in behavior and angiogenesis, and between said differences and metastatic tumor development in Lewis rats (Sajti et al., 2004). In relation to social stress, Amkraut and Solomon (1972) found that animals which engaged in spontaneous fighting behavior when housed in a group developed smaller virus-induced sarcoma tumors than their non-fighter counterparts. Vegas, Fano, Brain, Alonso and Azpíroz (2006) found that subjects inoculated with B16F10 melanoma which presented a behavioral strategy characterized by an absence of attack, low non-social exploration and high levels of defense/subordination and avoidance, developed more pulmonary metastases than those who presented a more active strategy. Furthermore, evidence exists to indicate that different neuroendocrine responses produced by stress, in association with different coping strategies (Koolhaas et al., 1999), have different immune consequences (Bartolomucci et al., 2001; Gasparotto, Ignacio, Lin, & Goncalves, 2002; Strauman, Woods, Schneider, Kwapit, & Coe, 2004).

Up until now, the differences regarding tumor development, associated with different strategies for coping with social stress, have mainly been studied in defeated animals which engage in predominantly submissive behavior. However, maintaining dominant status may also give rise to a stress response and extracts a high cost from the subject (Creel, 2001; McKittrick et al., 2000). Furthermore, it is known that one of the consequences of the immune activity is the secretion of proinflammatory cytokines by the peripheral immune cells. The action of these cytokines on the Central Nervous System (CNS) generates changes in behavior, the central monoaminergic metabolism and the activation of the hypothalamic-pituitary-adrenal axis (HPA) (Dantzer et al., 1996; Konsman, Parnet, & Dantzer, 2002). These behavioral changes are considered non-specific and are known as illness behavior. Similarly, tumor development produces changes in the cerebral metabolism similar to those produced by infection or injury (Chuluyan, Wolcott, Chervenak, & Dunn, 2000; Vegas, Beitia, Sánchez-Martín, Arregi, & Azpiroz, 2004). Consequently, it may also result in illness behavior, and may therefore alter the type of behavior manifested by subjects in response to social stress.

Based on these considerations, the objectives of this study were, firstly to study the effect of social stress on the development of melanoma tumor metastases in winner mice from dyadic confrontations, as well as the relationship between behavioral profiles prior to tumor inoculation, the coping strategies shown in response to social stress and tumor development; and secondly, to study the effect of tumor development itself on social behavior.

#### Materials and methods

##### Subjects and husbandry

Six week-old male outbred OF-1 mice (CRIFFA, Spain) were individually housed for 17 days in transparent plastic cages measuring  $24.5 \times 45.5 \times 15$  cm. Food and water were available ad libitum. The holding room was maintained at a constant temperature of 20 °C with a 12 h light/dark cycle (white lights on from 20:00 to 08:00 h) and at a relative humidity of 50-60%. All procedures involving mice were carried out according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 18 March 1986) as well as to related secondary and supplementary legislation.

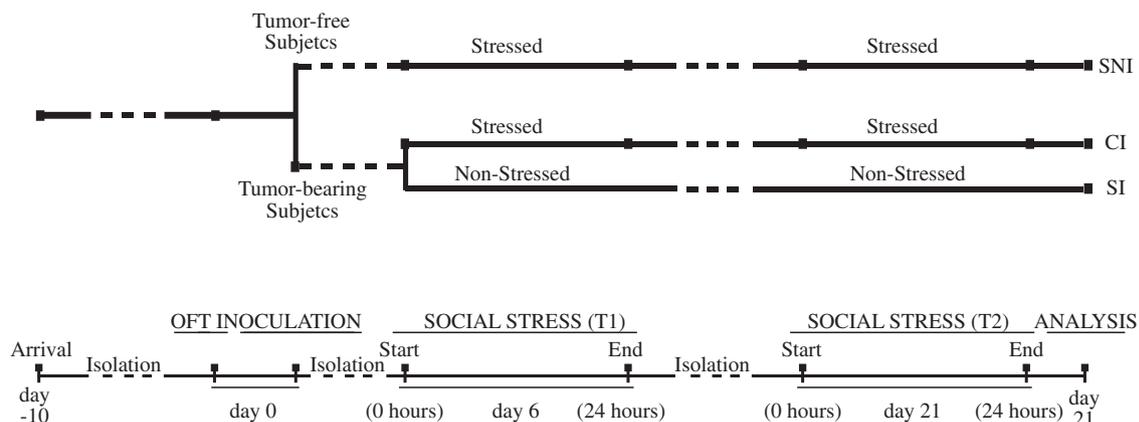
##### Experimental design

During the 10<sup>th</sup> day of isolation, subjects were submitted to an Open Field Test (OFT) and randomly allocated to two groups (figure 1). One group was inoculated with tumor cells (Inoculated group,  $n=46$ ) and the other with vehicle (Non-Inoculated group,  $n=10$ ). Animals inoculated with tumor cells were then divided into two new subgroups called the control-inoculated group (CI,  $n=8$ ) and the stress-inoculated group (SI,  $n=38$ ); all non inoculated animals were subjected to social stress (SNI,  $n=10$ ). Six days (Time 1, T1) and 21 days (Time 2, T2) after tumor inoculation, animals in the stressed group (both inoculated and non-inoculated) were exposed to the sensory contact social stress model (Kudryavtseva, Bakshtanovskaya, & Koryakina, 1991) for 24 h. With the aim of making the social stress stimulus equal for all animals, social interaction involved contact with anosmic intruder subjects. During this 24 h period, subjects were only exposed to direct physical interaction for three 5 min intervals, separated by two approximately 12 h periods. The rest of the time, anosmic mice were separated from the experimental subjects by perforated methacrylate barriers, which bisected the cage and allowed sensory (non-physical) contact outside the direct confrontation periods (Vegas, Fano, Brain, Alonso, & Azpiroz, 2006). The CI group remained in isolation during the entire 24 h period, but a methacrylate separator was introduced into the cages during these two intervals in order to monitor the effect of the separator itself and the resulting reduction in space. Between the first and second social interaction (T1 and T2), the animals remained individually housed in standard conditions.

One hour after the second 24 h social stress session (T2), subjects were put down and their lungs were infused with formal calcium. After several days in Bouin's solution, the upper lobe of the left lung was separated and the number of metastatic foci counted by a researcher who did not know to which group the animals belonged, using an Olympus SZ30 Microscope (Olympus, Japan).

##### Experimental tumor induction

Tumors were induced by B16F10 melanoma murine cells. The B16F10 cells were maintained in vitro by subculturing the tumor cells as described in previous works (Vegas et al., 2004).



**Figure 1.** Schematic representation of the experimental design. The animals were housed individually at all times, in standard conditions, except during the two 24-hour social interaction periods (6 and 21 days after tumor inoculation)

Melanoma adherent cells were detached by exposure to 0.02% EDTA for 5-8 min and washed three times in RPMI-1640 medium. The mice, pre-anesthetized with i.p. Nembutal (Sodium pentobarbital; 60 mg/kg), were injected i.v. with  $5 \times 10^4$  of viable B16F10 cells in 0.1 ml of medium into the lateral tail vein.

#### Behavioral assessment

**Open Field Test (OFT).** The OFT is a commonly used test for measures of general exploration and emotional reactivity (Ortet & Ibáñez, 1999). The OFT used consisted of a wooden box measuring  $80 \times 80 \times 50$  cm. The floor of the arena was divided into 64 equal squares. The animals were positioned in the corner of the OFT and were recorded using video cameras (Panasonic RX66, Japan) for 6 min, in order to analyze their behavior. After each test, the arena was cleaned three times with water, alcohol and, finally, water. Spontaneous activity (the number of times the mouse crossed the floor squares with both hind paws) and latency (time lapse for crossing the first square) were assessed using The Observer 3.0 (Noldus ITC, The Netherlands).

**Social Behavior.** The first two 5 min intervals of direct physical interaction (Time 1) and the last interaction prior to death (Time 2) were recorded using video cameras (Panasonic RX66, Japan) in order to analyze the behavior of the animals subjected to the sensory contact social stress model. Behavioral evaluation was carried out using The Observer 3.0 (Noldus ITC, The Netherlands), with a specific configuration based on the ethogram of the mouse developed by Brain, McAlliste and Walmsley (1989). The analysis of the behavioral variables was carried out by combining the two intervals into a single 10 min block for each subject.

#### Data analysis

All statistics involved SPSS 13.0 for Windows (SPSS Inc., USA) with the level of significance set at  $P < 0.05$ . Where parametric distributions occurred the data were analyzed using a one-way analysis of variance (ANOVA). When results were not normally distributed the non-parametric statistic Mann-Whitney U test was performed. A two factor ANOVA with repeated comparisons was used to investigate differences between the experimental groups and the social behavior evaluated to Time 1 and Time 2. To classify animals into behavioral subgroups, a hierarchical cluster analysis was employed using locomotor activity and latency as classifying variables (see the corresponding results section). To observe the relationship between passivity/activity and tumor development, a point biserial correlation analysis was used.

### Results

#### The effect of social stress on the metastatic development of pulmonary B16F10 melanoma cells

As shown in figure 2 subjects exposed to social stress had more pulmonary metastases than controls ( $U = 72.5$ ,  $P < 0.05$ ). No differences were observed between inoculated and non inoculated subjects in relation to social behavior assessed 6 days after inoculation (T1; figure 3a). However, 21 days after inoculation (T2; figure 3b), subjects inoculated with the tumor dedicated less time to digging ( $F_{1,45} = 19.116$ ,  $P < 0.001$ ) and exploration at a

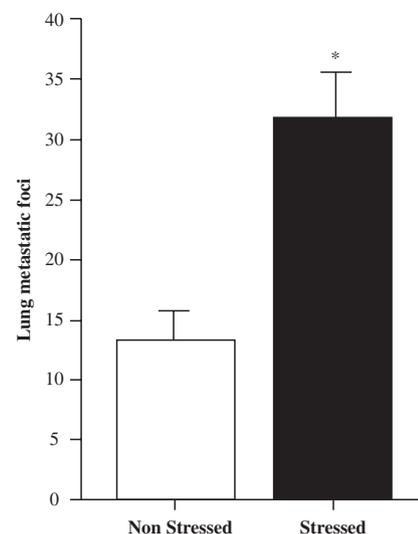
distance ( $F_{1,45} = 4.302$ ,  $P < 0.05$ ) and more time to body care ( $F_{1,45} = 4.762$ ,  $P < 0.05$ ). The analysis of the social behavior manifested by both inoculated and non inoculated animals 6 and 21 days after tumor inoculation (T1 vs. T2), indicated that at T2, the animals spent less time engaged in attack ( $F_{1,45} = 13.881$ ,  $P < 0.01$ ) and non social exploration behaviors ( $F_{1,45} = 60.281$ ,  $P < 0.001$ ) and more time engaged in avoidance ( $F_{1,45} = 185.305$ ,  $P < 0.001$ ), exploration at a distance ( $F_{1,45} = 14.030$ ,  $P < 0.01$ ); body care ( $F_{1,45} = 4.234$ ,  $P < 0.05$ ) and digging ( $F_{1,45} = 5.290$ ,  $P < 0.05$ ). Furthermore, subjects inoculated with experimental tumors showed decreased engagement in exploration at a distance: ( $F_{1,45} = 4.717$ ,  $P < 0.05$ ); and digging: ( $F_{1,45} = 4.564$ ,  $P < 0.05$ ) and an increase in time dedicated to body care ( $F_{1,45} = 4.529$ ,  $P < 0.05$ ).

#### Open field test

None of the three groups displayed any differences in the behaviors studied (locomotor activity:  $F_{2,53} = 0.476$ ,  $P = 0.624$ ; latency:  $F_{2,53} = 0.939$ ,  $P = 0.397$ ) (table 1). A cluster analysis using these two variables was carried out for all the animals. As a cut-off criterion, the inflection point was established at a distance equal to 5, resulting in two clusters. A multivariate discriminant analysis was performed (Wilk's Lambda method with step entry) to ensure the integrity of the groups derived from the cluster analysis. The discriminant model applied accounted for 96.4% of cases obtained by the cluster solution, confirming the statistical validity of these groups. As shown in figure 4, the subjects included in cluster one ( $n = 26$ ) were characterized by high locomotor activity and low latency (active behavior). On the other hand, subjects in cluster 2 ( $n = 30$ ) showed less locomotor activity and greater latency (passive behavior). The distribution of the subjects into different experimental groups (SI, SNI and CI) was homogenous (figure 5).

#### Behavior in the open field test and tumor development

Differences in pulmonary metastatic development were related to the different behaviors (active and passive behavior) shown in



**Figure 2.** Mean ( $\pm$ S.E.M.) pulmonary metastatic foci numbers in CI ( $n = 8$ ) and SI ( $n = 38$ ) groups, inoculated with B16F10 murine melanoma cells, 21 days after inoculation. \*  $P < 0.05$

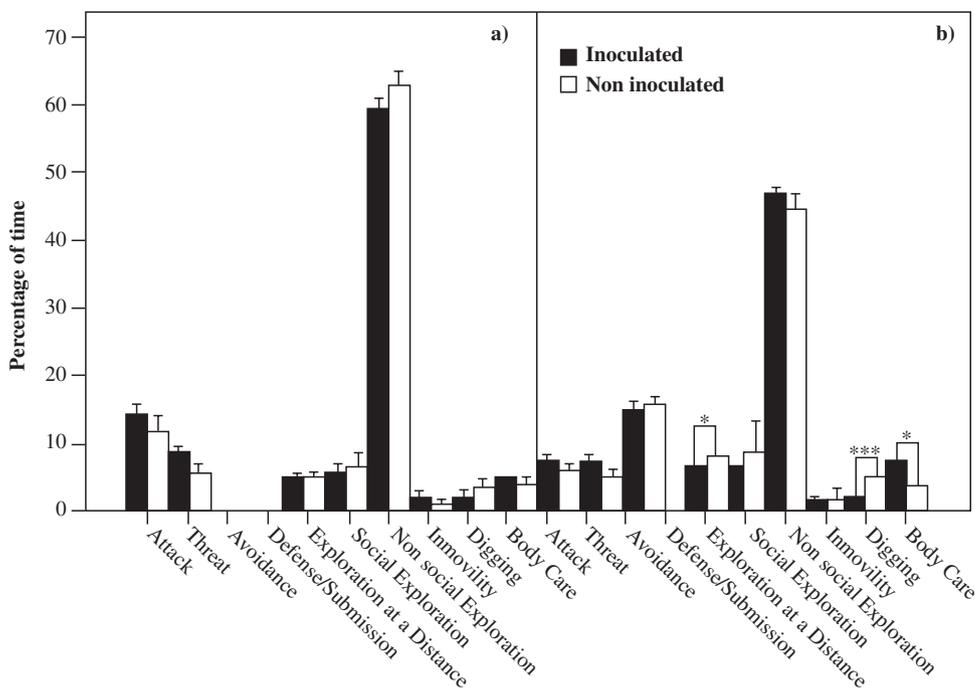


Figure 3. Percentage of time (mean ±S.E.M.) dedicated to each of the behavioral categories at T1(a) and T2(b), in male OF1 mice subjected to social stress. SI, (n= 37) vs. SNI (n= 10). \* P<0.05, \*\*\* P<0.001

the OFT (figure 6). The most passive subjects (Cluster 2, n= 25) showed greater tumor development than their more active counterparts (Cluster 1, n= 21) ( $F_{1,44}= 4.605, P<0.05$ ). Furthermore, a positive correlation was found between Cluster 2 (passive subjects) and tumor development ( $rpb= 0.304, t(44)= 2.117, P<0.05$ ). In relation to social behavior analyzed at T1, no significant differences were found between subjects classified as active in the OFT and those classified as passive. However, as shown in figure 7, the analysis carried out at T2 revealed that

active animals dedicated a greater amount of time to attack ( $F_{1,45}= 13.365, P<0.01$ ) and avoidance behavior ( $F_{1,45}= 5.953, P<0.05$ ) than their passive counterparts. Similarly, the analysis of the social behavior manifested by both inoculated and non inoculated animals 6 and 21 days after tumor inoculation (T1 vs. T2), indicated that at T2 subjects dedicated less time to attack ( $F_{1,45}= 22.675, P<0.001$ ) and non social exploration ( $F_{1,45}= 70.028, P<0.001$ ) behaviors and more time to body care ( $F_{1,45}= 14.509, P<0.01$ ), exploration at a distance ( $F_{1,45}= 8.374, P<0.01$ ) and

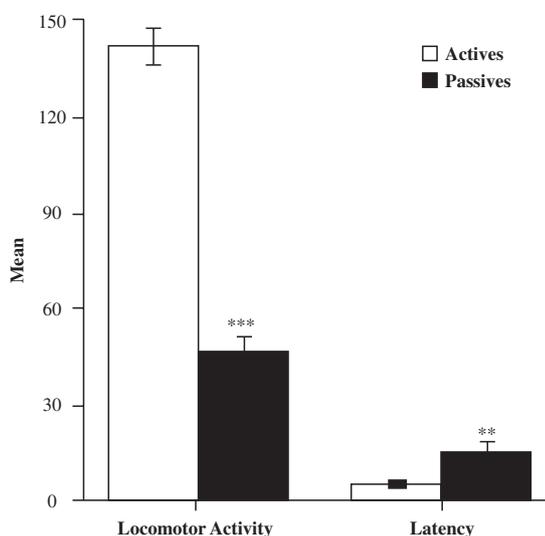


Figure 4. Mean (±S.E.M.) of the behavior observed in the Open Field Test (locomotor activity and latency), for subjects assigned to cluster 1 and cluster 2. \*\* P<0.01, \*\*\* P<0.001

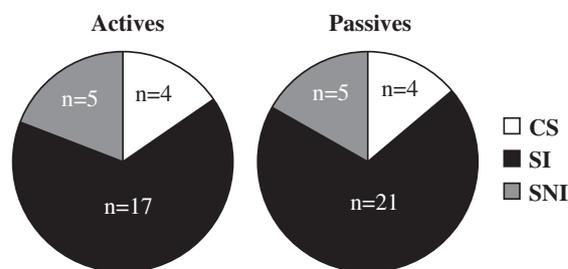


Figure 5. Distribution of subjects from groups SI, SNI and CI in each of the clusters. (Active and Passive)

Parameters	CI (n= 8)	SI (n= 38)	SNI (n= 10)
Locomotor activity	98,7±64,44	85,9±53,44	103,2±57,16
Latency	4,27±0,73	11,87±16,58	10,1±8,38

avoidance ( $F_{1,45} = 309.013, P < 0.001$ ); the interaction of the cluster factor was only evident for avoidance behavior, with active animals being those which dedicated a greater percentage of time to this behavior at T2 ( $F_{1,45} = 5.866, P < 0.05$ ).

Discussion

Our results reveal that winner animals present greater tumor development than their non stressed counterparts. These data are in agreement with those found by other authors using different social stress models (Stefanski & Ben-Eliyahu, 1996; Strange, Kerr, Andrews, Emerman, & Weinberg, 2000; Vegas, Fano, Brain, Alonso, & Azpiroz, 2006). Diverse studies have demonstrated that social stress produces a decrease in the activity of diverse parameters of the immune activity (Avitsur, Stara, Dhabhar, & Sheridan, 2002; Bartolomucci et al., 2001; Cacho et al., 2003; Stefanski & Engler, 1998) and in some cases, this immunosuppression is accompanied by greater tumor development (Stefanski & Ben-Eliyahu, 1996; Vegas, Fano, Brain, Alonso, & Azpiroz, 2006). Until now, the majority of existing data regarding the effects of social stress on tumor development have derived from the study of defeated animals, in which the physiological response to stress is mainly characterized by the activation of the HPA axis (Blanchard, McKittrick, & Blanchard, 2001; Martínez, Calvo-Torrente, & Pico-Alfonso, 1998). In our study, the experimental subjects are winner animals from a dyadic confrontation, and the greater tumor development observed can be attributed to the fact that maintaining dominant status also constitutes a source of stress that exacts an important organic cost (Bartolomucci et al., 2004, 2005; Sgoifo et al., 2005). Numerous studies indicate that the profile of dominant subjects or those animals which adopt a proactive style is characterized by a strong tendency to defend their territory and by behavioral activation (Fano, Sánchez-Martín, & Brain, 1997). Furthermore, this is related to sympathetic hyperactivity, a lower level of HPA axis reactivity and high levels of testosterone (Creel, 2001; Koolhaas et al., 1999). Thus, these factors may be involved in the increase in tumor development observed in our winner subjects. In accordance with this, diverse

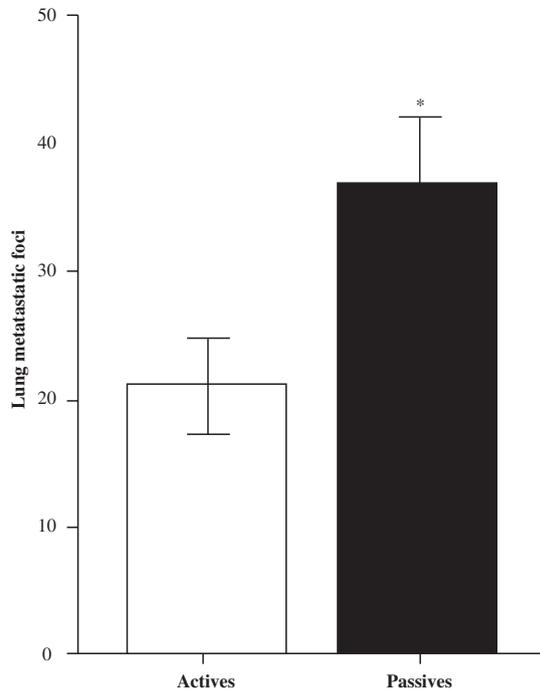


Figure 6. Mean (±S.E.M.) pulmonary metastatic foci numbers in all subjects [active (n= 21) and passive (n= 25)] inoculated with B16F10 murine melanoma cells, 21 days after inoculation. \* P<0.05

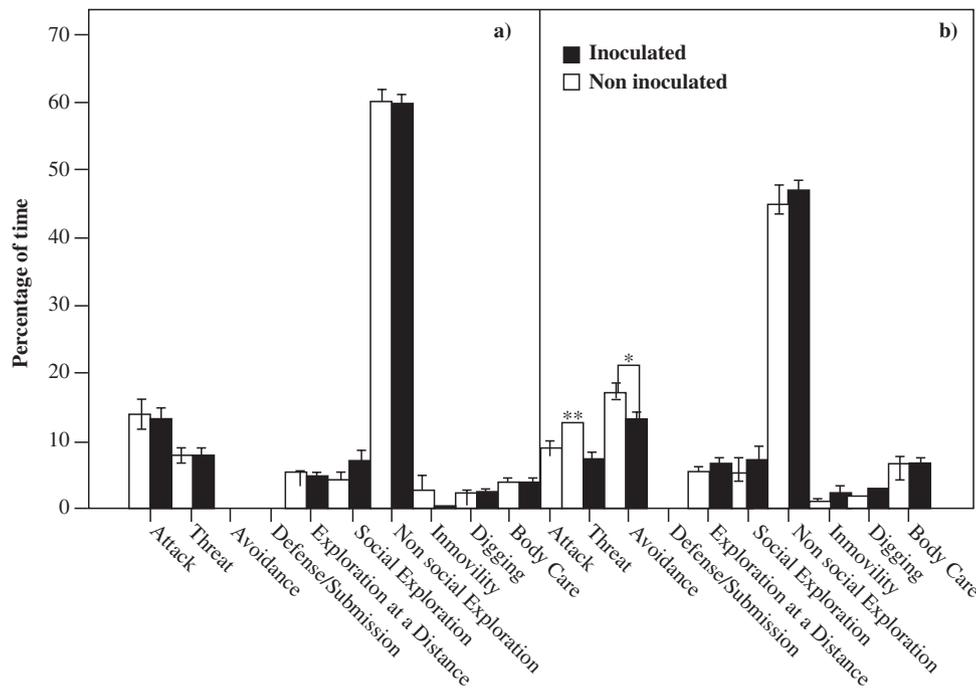


Figure 7. Percentage of time (mean ±S.E.M.) dedicated to each of the behavioral categories at T1(a) and T2(b), in male OF1 mice subjected to social stress. Active subjects (n= 22) vs. Passive subjects (n= 25). \* P<0.05, \*\* P<0.01

authors using  $\beta$ -adrenergic antagonists have found a greater tumor development mediated by adrenergic mechanisms, the elements possibly responsible for the alteration of the NK function (Stefanski & Ben-Eliyahu, 1996). Also, in our laboratory, we have found that the specific blocking of  $\beta$ -adrenergic receptors (nadolol) reduces tumor development in subjects exposed to this stress model (Vegas, Garmendia, & Azpiroz, 2006). Nevertheless, we cannot dismiss the possible influence of the other factors indicated above.

Bearing in mind the homogenous distribution of both clusters between the different experimental groups and the positive correlation between passivity and tumor development, our results suggest that animals which manifest different behavioral characteristics before being subjected to social stress and tumor inoculation, present differences in tumor development. Thus, it was observed that those subjects which presented a more passive behavioral profile in the open field test (Cluster 2) experienced greater tumor development than those which presented a more active behavioral profile (Cluster 1). In accordance with these results, Sajti et al. (2004) found more pulmonary metastatic foci larger than 2 mm, as well as greater angiogenesis in subcutaneous tumor implants (MADB 106) in rats characterized by their passivity in an open field situation. These data indicate that different personality characteristics may give rise to different physiological responses to stress.

In previous studies carried out in our laboratory, we found that different behavioral strategies in subordinate subjects manifested in response to social stress corresponded to differences in tumor development (Vegas, Fano, Brain, Alonso, & Azpiroz, 2006). Nevertheless, in this study, in which the experimental subjects were dominant animals, this relationship was not observed.

Nor did we observe any relationship between prior behavioral characteristics and the manifestation of different coping strategies for social stress in winner animals. This may be due to a general effect on the behavior of all subjects caused by the tumor development itself during its early phases. Some studies have observed changes in the brain neurochemistry of tumor-bearing mice, associated with hyperammonemia and reduced food intake (Chance, Cao, & Fischer, 1990; Chance, von Meterfeldt, & Fischer, 2003). However, in relation to social behavior, we failed to find any significant differences between inoculated and non inoculated subjects in any of the behavioral categories recorded at Time 1.

Nevertheless, in our procedures, which aimed to prevent injury to the experimental subjects and to ensure that the behavioral characteristics of the opponents were as homogenous as possible, we used anosmic subjects, which do not provoke such intense agonistic behavior in their opponents. This may have affected the possibility of observing a greater degree of behavioral variability in experimental subjects, since they were not faced with a particularly threatening challenge. Consequently, the failure to observe differences in tumor development between the coping

strategies employed by winner subjects in response to social stress may be the result of the type of social stress model used.

The second aim of this study was to observe the effects of tumor development itself on social behavior. To this end, a second aggressive confrontation was conducted 15 days after the first one, just before the animals were put down in order to examine the formation of pulmonary metastases. The results show a decrease in the amount of time dedicated by inoculated animals to exploration at a distance and digging, and an increase in the time dedicated to body care. Although we did not record the activity of the immune system and we cannot dismiss the possible effects of tumor growth itself, it is reasonable to assume that these effects were originated by an immune activation generated by the tumor. It is well known that the B16 melanoma has immunogenic effects (Houghton, Gold, & Blachere, 2001; Jensen et al., 1999). Although illness behavior has been characterized as a decrease in activity and an absence of interest in the subject's environment (Chuluyan et al., 2000; Dantzer et al., 1996; Vegas et al., 2004), the changes observed in our study do not coincide exactly with this definition. Nevertheless, changes in behaviors such as digging and exploration at a distance may indeed be interpreted as a decrease in the animal's interest in its environment. Furthermore, it is important to remember that the data relating to the behavior of the group of inoculated animals comprise data relating to individuals with different levels of tumor development (animals belonging to the active cluster and those belonging to the passive cluster). When differences in social behavior are observed separately for each group, those animals with greater tumor development show a significant decrease in attack and avoidance behaviors. These data coincide with those obtained by Cirulli, De Acetis and Alleva (1998) in which the administration of IL-1, resulted in a decrease in the aggressive components of agonistic behavior, even though defensive elements were not affected.

This lower level of attack and avoidance behavior shown by subjects with greater tumor development may indicate a change of strategy in response to social stress, which in turn may be considered a result of illness behavior.

In short, we can conclude that social stress in dominant subjects results in an increase in tumor development. This tumor development is greater in animals with a previously more passive behavioral profile and is not related to the behavioral strategies manifested by said subjects in response to social stress. Tumor development generates changes in social behavior during the final phases of development, with said changes being more evident in subjects with a more passive behavioral profile.

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