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Social Confrontation and Tumor Metastasis in Rats: Defeat and β-Adrenergic Mechanisms

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STEFANSKI, V. AND S. BEN-ELIYAHU. *Social confrontation and tumor metastasis in rats: Defeat and β-adrenergic mechanisms.* PHYSIOL BEHAV **60**(1) 277–282, 1996.—The effect of social confrontation on the susceptibility to metastatic development was studied in rats. An intruder male Fischer 344 (F344) was introduced to a male–female Long–Evans pair and the behavior was recorded during the first 30 min of a 7-h confrontation session. Mammary tumor cells (MADB106), syngeneic to the inbred F344 rat, were injected IV to the intruder 1 h after the beginning of the confrontation session, and the lung retention of tumor cells was determined 24 h later. In this tumor model, metastases develop only in the lungs. Retention of tumor cells and the consequent development of lung colonies are known to be highly controlled by the activity levels of natural killer cells during the first 24 h after tumor inoculation but not later. Twenty of the 21 intruders were attacked by resident males and 19 displayed submissive behavior. A significant increase in lung tumor retention was evident in intruders compared to both control groups: home cage and new environment. The magnitude of this increase was higher in intruders that frequently displayed submissive behavior (indicating social defeat). Pretreatment with the β-adrenergic antagonist, butoxamine, reduced the effects of social confrontation by approximately 50%, and adrenal demedullation almost abolished it without significantly affecting the social interaction. These findings suggest that the nature of intruder–resident interaction, rather than being subdominant or exposure to an unfamiliar environment, has a marked influence on the intruder's susceptibility to metastatic development. These effects of social confrontation seem to be mediated by adrenergic mechanisms, possibly via adrenergic influence on NK function and distribution.

Confrontation Stress Cancer Metastasis Natural killer β-Adrenergic
Adrenal demedullation Aggression Behavior MADB106

AMPLE evidence suggests that the immune and the neuroendocrine systems interact and regulate the activity of each other (1). The impact of nonsocial "stressors," such as electroshocks, on immunity and health have been frequently shown, and psychological factors have been suggested to play a role in some of these stress paradigms (34,46). Aggressive social interactions in mammals are often associated with elevated levels of glucocorticoids and cathecholamines (26,29), hormones that are known to affect certain aspects of immunity [e.g., (11,15,28,32)]. Nevertheless, the relationship between behavioral factors, neuroendocrine responses, and immune competence have only recently been addressed more systematically (13). Male Long–Evans rats, classified as defeated in resident–intruder interactions, produced a significantly lower antibody response after keyhole limpet hemocyanin (KLH) challenge, compared to not defeated subdominant rats or control subjects (18). Using guinea pigs, we demonstrated (47,49) that complement activity was low when social situation was characterized by an unstable dominance relationship. Investigating the effects of social defeat and submissiveness, several studies indicate that psychosocial, rather than physical, aspects of social interaction are associated with physiological reactions generally considered as "stress" responses (29,43). For example, defeat experience, rather than receiving bites, is correlated with stress-induced analgesia in mice (38).

The impact of stressful conditions on natural killer (NK) cell activity (39) and metastatic spread (6) has been documented. The

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outcome of social interactions also might be of clinical relevance. Assessing experimentally induced tumor growth and NK cell activity in female Syrian hamsters, a correlation between individual behavioral patterns and tumor development was evident: a more active pattern of behavior was associated with worse tumor outcome compared to an "inactivity (dominance)" behavioral pattern (51). The impact of previous resident–intruder experience on survival time following pre-B-cell tumor cell inoculation was investigated in rats (14). Resident males had a shorter survival time than the intruders. The effect on surival time was independent of the social status established during the fights, but related to the numbers of clinches. In early studies, Amkraut and Solomon (2) demonstrated that smaller sarcoma virus-induced tumors occur in female rats that display fighting behavior. Human studies also suggested an association between personality/behavioral traits and susceptibility to malignant growth (16,20,45,50). The present study assesses the role of specific psychosocial factors and the involvement of adrenergic mechanisms in affecting host resistance to tumor metastasis in the context of social confrontations. Specifically, behavior differences of the intruder males during confrontation were monitored and related to individual resistance to tumor development. Although laboratory rats show relatively little spontaneous fighting behavior in established social environments, introduction of unfamiliar males (intruders) into established pairs usually results in aggressive behavior of the "resident" male towards the intruder (12,33,37), especially when an aggressive strain is used. Resident–intruder confrontation in laboratory rats contains most of the agonistic behaviors of free-living subjects (37), and can be used to study various outcomes of social confrontation. Most commonly, attacks by the resident male upon the intruder start within the first 5 min after introduction of the intruder, which displays a variety of behavioral patterns clearly indicating subdominance (12,19,37).

The MADB106 tumor cell line used in this study is a mammary adenocarcinoma syngeneic to the F344 rat. Following IV injection, this tumor metastasizes only in the lungs, and its clearance from the lungs and the number of lung metastases established are highly controlled by the activity levels of NK cells (4,5,7). NK cells are known to be important in controlling the metastatic process in general (21,23–25,53) and specifically in relation to MADB106 tumor used in the present study (4,5,7). A complete depletion of NK cells causes approximately 100-fold increase in lung tumor retention, assessed 24 h following tumor injection, and a similar increase in the number of lung metastases counted weeks later (7). Thus, this is a good model for studying cancer metastasis and for assessing the involvement of NK cells in mediating alteration in the host's antimetastatic activity. In this study we chose to assess the efficiency of the metastasis process by determing lung tumor retention 24 h following MADB106 inoculation. The clearance of MADB106 tumor cells from the lungs (lung clearance) is a cumulative process that, within each rat, reachs a plateau level between 18–24 h following tumor administration. The level of this plateau is highly predictive of the number of lung metastases that can be counted a few weeks later. During this first 24 h, but not later, NK cells have a crucial role in determining the efficiency of lung clearance. Therefore, the assessment of lung tumor retention at 24 h following inoculation is indicative of the overall efficiency of the metastatic process throughout this critical period, and is optimal in reflecting alterations in the in vivo levels of NK cell activity.

Because NK activity and distribution have been shown to be affected by catecholamines (8–10,27), the role of catecholamines in mediating the effects of confrontation on metastatic development was assessed by using the β-adrenergic antagonist, butoxamine, and by using adrenal-demedullated rats.

METHOD

Animals and Housing Conditions

Upon arrival, Fischer 344 (F344) male rats were randomly divided into three groups: "intruders," home cage controls, and new environment controls. All animals, including adrenal-demedullated (ADM) and sham-operated (SHAM) male F344 rats, were purchased from Harlan–Sprague–Dawley, San Diego, at the age of 6 weeks and maintained under standard conditions. Long–Evans rats were used as "residents" as this strain is more aggressive in the context of resident–intruder confrontations. At least 25 days prior to the experiment, F344 and Long–Evans male–female pairs were established (with age- and strain-matched females) to allow development of social relations. Offspring were removed within 24 h after birth. A minimum period of 2 days after birth was allowed before males of these pairs were used in confrontations. All animals were 3–4 months old at the time of the experiment and were kept under a 12:12 h light:dark cycle (light from 0800 to 2000 h) in $40 \times 30 \times 60$ cm stainless steel cages, covered with a wire mesh that allowed behavioral observation. Room temperature was $20 \pm 1°C$; commercial rat diet and water were available ad lib.

MADB106 Tumor Cells

MADB106 is a selected variant cell line obtained from a pulmonary metastasis of a mammary adenocarcinoma (MADB100) chemically induced in the inbred Fischer 344 rat (5). MADB106 cells were maintained in an humidified incubator at 5% CO_2 and 37°C in monolayer cultures in complete media [RPMI 1640 media (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated FBS, 45 U penicillin-G/ml, 0.045 mg streptomycin/ml, 2 m*M* L-glutamine, 0.1 m*M* nonessential amino acids, and 1 m*M* sodium pyruvate]. Cells were separated from the flask (Falcon 3023) using 0.25% trypsin. Radiolabeling of tumor cells and lung clearance assessment for DNA radiolabeling of tumor cells was conducted by adding 0.4 μCi of ^{125}Iododeoxyuridine(^{125}IDUR) (ICN Radiomedicals, Irvine, CA) per ml complete media to the growing cell culture 1 day before harvesting the cells for injection. Rats were lightly anesthetized with halothane, and 10^5 (approximately 4×10^5/kg) ^{125}IDUR-labeled MADB106 tumor cells in 0.5 ml PBS were injected into the tail vein. The anesthesia and injection procedures take approximately 1–2 min, followed by immediate awakening of the animal. Twenty-four hours later, rats were euthanized with halothane, lungs were removed, and their radioactive content was measured in a gamma counter. Percent radioactivity retained in lungs is the ratio between radioactivity counted in the lungs and total radioactivity of the radiolabeled MADB106 tumor cells injected. Our previous studies with these radiolabeled tumor cells indicate that when these cells are destroyed the great majority of radioactive molecules are excreted in the urine within a few hours (7).

Experimental Design and Confrontation Procedure

Thirty minutes before confrontation, F344 males were injected in their home cage with either saline or butoxamine (25 mg/kg) (eight rats in home cages were handled without injection). At the beginning of a 7-h confrontation session, F344 males were removed from their home cage and introduced into residents' cages each containing a male–female Long–Evans pair. The behavior of the animals was recorded as detailed in the Behavioral Observation section. One hour after the beginning of confrontation, the intruder (or corresponding control subject) was

removed for approximately 3 min from the residents' cage (or control cage) for injection of radioactive-labeled MADB106 cells (see Injection Procedures) and for a second injection of either saline or butoxamine (25 mg/kg). Three types of controls were used: home cage control rats, which remained in their cages and were either injected with saline or received no injection, and new environment control rats that were moved for the period of 7 h to an empty cage (that was recently populated by a Long–Evans pair and contained their bedding materials and odors) and were injected with saline. In each experiment conducted, control animals received all injections at the same time intervals as confrontation animals, unless specifically noted. All experiments were conducted at the beginning of the light period (max. time difference between first and last confrontation was 75 min). Timings and order of all injections were counterbalanced between confrontation and control groups. Twenty-four hours after tumor injection, intruders and control animals were scarified and lungs removed for determining lung retention of tumor cells. Rats were examined for possible bites at the time of tumor injection and before sacrificing. In all confrontations only one animal had to be separated before the end of the 7-h session and was excluded from further analysis. The experiments involving butoxamine were conducted in two replications a week apart. In the replicate we used eight home cage controls (four without saline, four with saline injection), six new environment controls, and 20 intruders (of which six were treated with butoxamine and 14 with saline). In the second replicate we used 9 (4,5), 0, and 14 (7,7) rats, respectively. The experiment involving ADM rats did not involve any injection other than the tumor cells and included six sham-operated home cage controls, six sham-operated intruders, nine ADM home cage controls, and six ADM intruders. We did not use control groups treated with butoxamine because in all previous studies we conducted, butoxamine had no significant effect on nonstressed control groups with respect to lung clearance of the MADB106 (8,9), and in the current study we used both ADM and sham-operated rats in control conditions.

Behavioral Observation

The behavior of all animals of each confrontation group was continously observed and quantitatively analyzed for the first 30 min of the confrontation session beginning with the introduction of the F344 male into the residents' cage. All animals in each cage were observed simultaneously and their behavior recorded using an abbreviation system. After an initial phase of vigorous attacks lasting for about 15–20 min, the frequency of resident attacks usually declined significantly. After this initial period attacks occurred only sporadically. From the end of the 30-min observation period until the end of the confrontation period (7 h) all confrontation groups were checked frequently to detect any change in dominance relationship or possible injury. Nomination of behavioral elements, mainly of agonistical context, follows the literature (19,36). Behavioral elements indicating subdominance are: upright posture with head back and nose pointed vertically (defensive upright); sideway posture with broadside orientation close to the opponent with head going outward (defensive sideway); full defensive posture, the animal is laying motionlessly on the back with ventral surface exposed to the opponent (this behavioral pattern is generally considered as an indicator of defeat in rats (12,36); attack, quick run, or jump orientated towards the opponent, physical contact with or without bite may occur. In all groups investigated, the vast majority of agonistic interaction occurred between the males. If, as in some groups, agonistic encounters occurred between the F344 intruder males and the Long–Evans females, the interactions were of a lower intensity. All behavioral numbers reported in the Results section are based on male–male encounters.

RESULTS

The Effects of Confrontation and Butoxamine Treatment on MADB106 Lung Tumor Retention

The 7-h confrontation session of 21 Fischer 344 rats with resident Long–Evans pairs resulted in about a threefold increase of radioactivity retained in the lungs (reduced lung clearance) compared to home cage and new environment controls (Fig. 1). Application of the β-adrenergic antagonist, butoxamine, before and during confrontation to 13 additional intruders reduced this effect by 50%, as evident from Fig. 1. ANOVA indicated significant group differences, $F(4, 52)$, $p = 0.017$. A post hoc comparison between single groups revealed significant differences between intruder males and both home cage and new environment-exposed F344 males (controls). Lung clearance of the butoxamine-treated intruders did not differ significantly from control animals as well as from nontreated intruder males. All 34 intruder males (regardless of butoxamine treatment) became subdominant as indicated by the displaying of either full defensive posture and/or defensive upright. Numbers of the behavioral elements defensive upright, defensive sideway, full defensive posture, and number of attacks received did not differ between nontreated and butoxamine-treated intruder F344 males (t-test, NS).

The Effects of Adrenal Demedullation

Adrenal demedullation abolished the effects of confrontation on tumor retention. Two-way ANOVA revealed a significant interaction between ADM and confrontation, $F(3, 23)$, $p = 0.007$. Whereas in sham-operated rats confrontation caused a significant increase in tumor retention (intruders vs. home cage controls, Fisher PLSD, $p < 0.05$, Fig. 2), no such differences were evident in ADM rats. No significant behavioral differences (in the agonistic elements recorded in this study) were observed between sham and ADM males during the first 30 min of the confrontation session (t-test, NS).

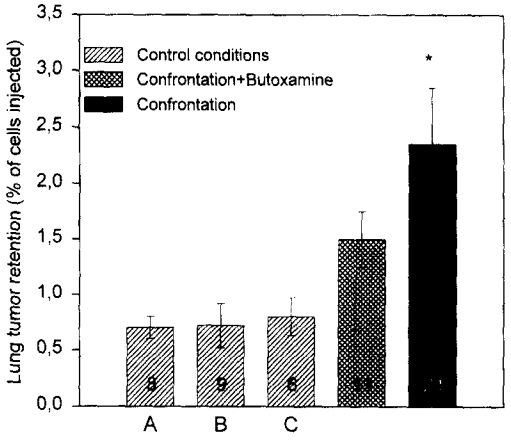

Fig. 1. Mean of percent radiolabled tumor cells (±SEM) retained in the lungs in F344 intruder and control males 24 h after the beginning of a 7-h confrontation session. All rats received saline or butoxamine injections except rats from control condition A group (home cage that were not injected at all). Control condition B is home cage; control conditon C is new environment. Number of animals are indicated at the bottom of each column. * $p < 0.05$, intruders (confrontation) vs. control condition 1, 2, and 3 (Fisher PLSD); all others NS.

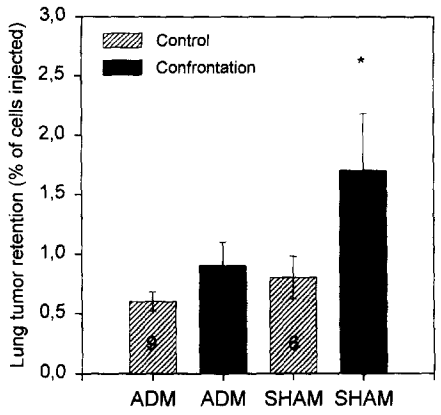

FIG. 2. Mean of percent radiolabled tumor cells (±SEM) retained in the lungs in ADM and SHAM F344 males 24 h after the beginning of a 7-h confrontation session or control condition. Number of animals are indicated at the bottom of each column. SHAM confrontation is significantly higher than all other groups (Fisher PLSD, $p < 0.05$); all other comparisons NS.

Individual Behavior Differences and MADB106 Tumor Retention

The magnitude of the increase in tumor retention in intruder males was strongly correlated with the nature of the agonistical interaction in "normal" rats. These normal rats received no butoxamine treatment and were not ADM or sham operated. Behavioral data (based on the 30-min observation period immediately after introduction) and tumor retention of these 21 normal intruder males were analyzed by stepwise multiple regression and correlation. The number of full defensive posture ($r = 0.646$, $p = 0.002$), the number of times being attacked by the resident male ($r = 0.830$, $p < 0.001$), and the number of bites received ($r = 0.615$, $p = 0.003$) correlate highly and significantly with radioactivity retained in the lungs of intruder males. In contrast, defensive sideway or defensive upright did not correlate significantly (Fig. 3). The indicator being attacked alone predicts 69.0% of the variance in this model, $F(1, 19) = 42.24$, $p < 0.001$. To eliminate factors related to "physical injury," in a second regression model seven intruders (including the one with highest lung tumor retention, cf. Fig. 3) that received bites were excluded. Similar to the previous model, full defensive posture ($r = 0.601$, $p = 0.023$) and being attacked ($r = 0.579$, $p = 0.03$) correlate significantly with lung clearance. A separate analysis of butoxamine-treated intruders indicated no significant relationship between individual behavior and lung tumor retention, as could be expected provided the decrease in the lung tumor retention in butoxamine-treated rats. (Note that average lung tumor retention of butoxamine-treated animals was reduced by approx. 50% and did not significantly differ from control subjects, cf. Fig. 1).

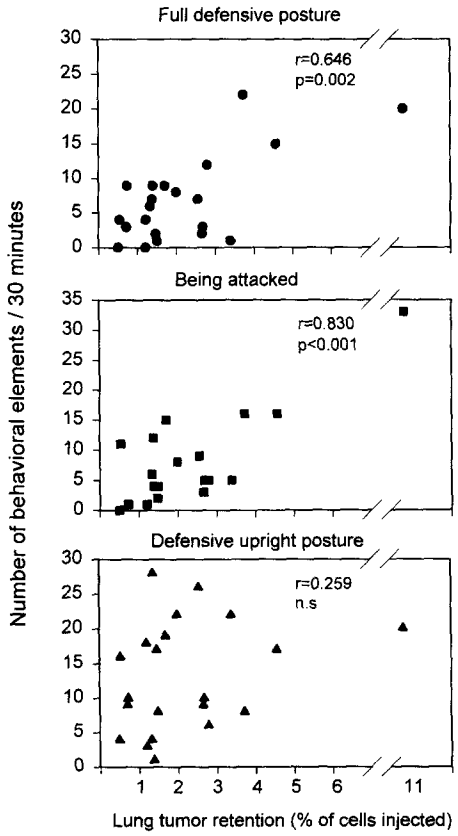

FIG. 3. Relationship between behavior displayed by 21 intruder males during the first 30 min of the 7-h confrontation session and percent of radiolabeled tumor cells retained in the lungs 24 h after the beginning of confrontation.

DISCUSSION

A 7-h exposure of male Fischer 344 (F344) rats to an established pair of Long–Evans rats results in an approximately threefold increase in the lung retention of tumor cells compared to new environment and home cage controls. New environment control animals showed similar levels of lung clearance as home cage control rats. Thus, interaction between the conspecifics, rather than exposure to an unfamiliar environment, is associated with a decrease in the host's antimetastatic activity. Importantly, the intruders' ability to resist MADB106 metastasis (i.e., lung clearance) was strongly associated with the nature of the intruder–resident interaction. Although all intruder rats became subdominant, lung clearance was especially poor in intruders that frequently displayed full defensive postures or/and that were repeatedly attacked by the (dominant) resident males. Lung clearance corresponded almost to control levels in intruders, which rarely displayed full defensive postures and were either ignored or approached less aggressively by resident males. Intruder males that frequently displayed full defensive postures can certainly be considered as socially defeated. Therefore, it can be suggested that the nature of the social interaction, rather than subdominance or the event of confrontation itself, influences physiological outcomes in the context of social confrontations. The decreased resistance to tumor metastasis in these defeated males is in accordance with other studies suggesting that the condition of social defeat is associated with major immunological changes, such as altered numbers and ratios of T cells (14), differential effects on T helper cell subsets (48), decreased complement activity (49), or reduced IgG antibody production (18).

Confronted and control animals may differ in their motor activity or other nonsocial factors (such as food or water consumption). However, we argue that the contribution of such nonsocial factors to the effects of social confrontation on lung clearance is small relative to the role played by psychosocial factors under such stressful social conditions. First, lung clearance was strongly associated with behavioral indicators of social defeat (full defensive posture), but not with other subdominat behavioral elements (such as defensive upright or defensive

sideway), and seem unlikely to be associated with motor activity. All confronted animals moved frequently and many intruder males that rarely displayed full defensive postures had good lung clearance, dispite displaying other subdominant behavioral elements frequently (such as upright posture). Second, the size of the effect of social confrontation on tumor retention is approximately 10-fold larger than the effect of a 12-h starvation period or a day of isolation, manipulations that we have studied in the past using this tumor model (7,40,41). Third, the effects of social confrontation were attenuated or blocked by the adrenergic antagonist butoxamine and by adrenal demedullation (ADM). These manipulations did not alter the observed physical behavior of the individuals significantly, but blocked the effects of social confrontation on lung clearance (probably by reducing certain aspects of sympathetic activation). Thus, this dissociation between the effects of these manipulation on the physical aspects of behavior and on lung clearance suggest that changes in motor activity are less important than stress-related changes in sympathetic activation in mediating the effects of social confrontation on lung clearance. Finally, the present study provides statistical evidence to support the suggestion that psychosocial, rather than the physical, aspects of confrontation correlate with alterations in the intruders' capability to resist tumor development. Using a multiple stepwise regression model, being attacked and full defensive posture correlate better with lung clearance than number of times being bitten. Moreover, even when the minority of males that received bites were excluded from analysis, both behavioral measures still correlated significantly with lung clearance. It is noteworthy in this respect that physical aspects of injury might affect lung clearance. However, it is clear that "being bitten" also characterizes an extremely psychosocially stressful condition.

The majority of the intruders were frequently attacked by the dominant resident males despite displaying submissive behavior (full defensive posture), a behavioral "strategy" usually successful in preventing further attacks in established social colonies (3). Therefore, and because housing conditions did not provide the intruder with the opportunity to escape, their social situation can be characterized as one with a low level of control. It could be hypothesized that the lack of controllability perceived by the intruder is an important factor determining the physiological consequences we report. Other studies have also suggested that conditions characterized by uncontrollability or unpredictability are associated with drastic physiological consequences such as gastric ulceration (52), reduced lymphocyte proliferation (31), decreased complement activity (49), or reduced IgG antibody production following KLH challenge (18).

In a study using pre-B-cell tumors, Bohus et al. (14) also indicated a relationship between behavior and tumor outcome in rats. Wistar males that were frequently involved in intensive interactions during confrontation ("clinch") showed a longer survival time. However, because of various methodological differences (duration and numbers of confrontation, classification of fight intensity, tumor type, rat strain, etc.), data from both studies cannot be easily compared.

Resident–intruder confrontations in murine species are correlated with elevated levels of catecholamines (22), corticosteriods (42), and endogenous opiods (38) in the defeated male. These hormones are also known to affect NK cell activity and NK body distribution [glucocorticoids (15,28,35); corticotropin-releasing factor (30); epinephrine (27); opioids (17,40)]. The partial blockade of the reported effects of social confrontation by the adrenergic antagonist butoxamine [together with the lack of its effects on baseline levels of lung clearance established in our previous studies (8,9)], suggests the involvement of cathechoaminergic mechanisms in mediating the effects of social defeat on tumor metastasis. This suggestion is further supported by the complete blockade of the effects of social confrontation in ADM intruders and the lack of effects of ADM in control condition evident in the current study. Because the behavior of butoxamine-treated and ADM intruders, as well as the response of the residents towards them, did not differ from untreated intruders, it is unlikely that the perception or the nature of the social situation was significantly impaired by these manipulations. Taken together with the critical role played by LGL/NK cells in controlling the lung clearance of these tumor cells (4,5,7), and the known influences of catecholamines on LGL/NK cell adhesion to epithelial cells, migration patterns, and activity levels, our findings suggest that LGL/NK cells might mediate the observed effect on susceptibility to metastasis. This suggestion is currently under investigation.

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