

## EVIDENCE THAT STRESS AND SURGICAL INTERVENTIONS PROMOTE TUMOR DEVELOPMENT BY SUPPRESSING NATURAL KILLER CELL ACTIVITY

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Stress and surgery have been suggested to compromise host resistance to infectious and malignant diseases in experimental and clinical settings. Because stress affects numerous physiological systems, the role of the immune system in mediating such effects is unclear. In the current study, we assessed the degree to which stress-induced alterations in natural killer (NK) cell activity underlie increased susceptibility to tumor development in F344 rats. Two stress paradigms were used: forced swim and abdominal surgery. Host resistance to tumor development was studied using 3 tumor models syngeneic to inbred F344 rats: CRNK-16 leukemia and the MADB106 mammary adenocarcinoma, both sensitive to NK activity, and the NK-insensitive C4047 colon cancer. Swim stress increased CRNK-16-associated mortality and metastatic development of MADB106 but not metastasis of C4047 cells. In both stress paradigms, stress suppressed NK activity (NKA) for a duration that paralleled its metastasis-enhancing effects on the MADB106 tumor. *In vivo* depletion of large granular lymphocyte/NK cells abolished the metastasis-enhancing effects of swim stress but not of surgical stress. Our findings indicate that stress-induced suppression of NKA is sufficient to cause enhanced tumor development. Under certain stressful conditions, suppression of NKA is the primary mediator of the tumor-enhancing effects of stress, while under other conditions, additional factors play a significant role. Clinical circumstances in which surgical stress may induce enhanced metastatic growth are discussed. *Int. J. Cancer* 80:880–888, 1999.

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Exposure to conditions of stress, such as surgery, death of a spouse, or other traumatic life events, has been associated with elevated rates of infectious disease, malignant growth and mortality (Biondi and Zannino, 1997; Cohen and Herbert, 1996; Helsing and Szklo, 1981). Both human and animal studies have indicated that different stressors can either increase or decrease indices of immuno-competence (Ader *et al.*, 1991; Andersen *et al.*, 1998; Ben-Eliyahu *et al.*, 1991; Shakhhar and Ben-Eliyahu, 1998). Similarly, stress has been shown to both augment and suppress tumor development in animal models (Kanno *et al.*, 1997; Sklar and Anisman, 1981). Whether stressful conditions are associated with an increase or a decrease in immunity or disease appears to be determined, on the one hand, by the physical and psychological characteristics of the stressor, such as chronicity, severity, controllability or predictability, and, on the other hand, by specific characteristics of the host, such as genetic makeup, personality traits or history of exposure to various environmental conditions (Ader *et al.*, 1991).

Although stress has been reported to decrease various immune indices, the extent to which the deleterious effects of stress on infectious disease and tumor development can be accounted for by this immune suppression is unclear (Cohen and Herbert, 1996). The response of the host to stressful conditions involves numerous physiological mechanisms other than the immune system, which can also affect morbidity. For example, hormone levels, growth factors, vascular permeability, blood pressure and glucose availability are affected by stress and, irrespective of their potential impact on immune competence, have been suggested to influence the progression of infectious and malignant diseases (Weiner, 1992). Moreover, the extent to which the immune system provides tumor surveillance, especially in humans, is unknown (Green, 1993). In

establishing a relationship between immune suppression and increased rate of malignancy, one encounters a major obstacle. Whereas infection may occur within days of stress onset or immune suppression, a long and variable interval is expected between stress-induced immune suppression and the detection of potentially related malignancies. Therefore, elucidating causal relationships between stress, immune suppression and increased rate of malignancy is a difficult task, especially in humans.

Whereas the outbreak of latent or opportunistic infections following surgery or other stressors is clinically well documented and considered to be a major life risk (Holzheimer *et al.*, 1997), the impact of such stressors on tumor development has not been clinically determined. Specifically, malignant development detected up to 5 years following surgical excision of a tumor is routinely considered to be metastatic growth of the primary tumor. The role of peri-surgical stress and immune suppression in promoting metastatic growth is unknown. Indeed, potential relationships of this sort are particularly relevant when a metastasizing tumor is surgically removed (*i.e.*, in patients with positive lymph nodes). The common prophylactic use of antibiotics with surgery would clearly be ineffective in combating tumor development and viral infection; thus, the elucidation of mechanisms underlying the effects of stress and surgery on disease progression, specifically the role of the immune system, would be instructive in developing procedures to minimize the possible deleterious effects of stress.

Among the immune mechanisms controlling malignancies and viral infection are natural killer (NK) cells, a subpopulation of leukocytes known to spontaneously recognize and kill a variety of tumor and virally infected cells *in vitro* (Herberman and Ortaldo, 1981; Moretta *et al.*, 1994). Animal studies have shown that NK cells play an important role in controlling the development of both leukemia and solid tumors (Brittenden *et al.*, 1996), especially during the metastatic process of various tumor types (Barlozzari *et al.*, 1983, 1985; Ben-Eliyahu and Page, 1992; Ben-Eliyahu *et al.*, 1991; Gorelik *et al.*, 1982; Hanna, 1985; Wiltrout *et al.*, 1985). Human studies have found a correlation between levels of NK activity (NKA) and susceptibility to several different types of cancer. For example, patients displaying lower levels of NKA (*e.g.*, Chediak-Higashi syndrome, X-linked lymphoproliferative syndrome and major psychotic depression) have also been reported to show a higher incidence of cancer (Brittenden *et al.*, 1996). Higher levels of NKA at the time of tumor removal have been associated with good prognosis following excision of various types of cancer, including breast cancer (Levy *et al.*, 1985; Schantz *et al.*, 1987; Taketomi *et al.*, 1998).

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In the current study, we sought to determine the relative importance of stress-induced suppression of NKA in mediating the effects of stress on tumor development. To this end, we first compared the time course of stress-induced NK suppression (measured *in vitro*) with the time course of stress-induced enhancement of tumor development. This comparison was conducted using a swim stress paradigm, inducing relatively short-term stress effects, and a surgical stress paradigm, characterized by longer-lasting stress effects. Further, we compared the impact of stress on NK-sensitive processes of metastatic development with its impact on NK-insensitive processes. Finally, using both stress paradigms, we compared the effects of stress on tumor development in normal rats with those in rats selectively depleted of NK cells.

## MATERIAL AND METHODS

### Animals

Fischer 344 male rats (Harlan, Jerusalem, Israel; Harlan Sprague-Dawley, Indianapolis, IN) were housed 4 in a cage with free access to food and water. Animals were acclimatized for at least 4 weeks prior to the beginning of experimentation and were 14 to 20 weeks old at that time. In any given experiment, all animals were of the same age and housed under the same conditions. Experiments were conducted during the first half of the dark phase of a 12-hr light/dark cycle, unless specified otherwise.

### Swim stress

A weight of 25 g/kg of body weight was attached to the tails of stressed rats. Each stressed rat was then placed for 3 min in a tank containing water 35 cm deep at a temperature of 37°C, followed by a 3-min rest period. This procedure was repeated 5 times successively. All rats housed in a cage either served as controls or were stressed. Rats from the control groups were left undisturbed in their cages.

### Surgical stress

The laparotomy procedure has been described elsewhere (Page *et al.*, 1994). Briefly, rats were anesthetized with 2.5% halothane, and a 4-cm midline abdominal incision was made. The intestine was externalized for 4 min, during which time it was kept moisturized and gently rubbed with a gauze pad. The intestine was then returned to the abdominal cavity and the wound sutured.

### Splenic and blood mononuclear NKA assay

This standard 4-hr cytotoxicity procedure assesses anti-tumor NKA per splenocyte or per blood mononuclear cell.

**Preparation of effector cells.** Rats were euthanized with halothane for spleen removal and for blood withdrawal *via* right ventricular puncture. Blood was heparinized upon removal (250 U/ml of blood in 0.5 ml saline), then centrifuged at 1,500 g for 20 min in combination with 120% Sepracell (Sepratech, Oklahoma City, OK). Mononuclear cells were then harvested from the uppermost layer (20% of overall volume) and washed twice in PBS supplemented with 0.1% BSA. Simultaneously, spleens were dissociated into a single-cell suspension using a 7-ml TenBroeck tissue grinder (Corning 7727, Corning, NY). Spleen cells were then washed twice in PBS and filtered through nylon mesh. Blood mononuclear cells and spleen leukocytes were then counted and their concentrations adjusted in complete medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 µg/ml gentamicin, 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate) to achieve the E:T ratios described below.

**Radiolabeling of YAC-1 target cells.** YAC-1 cells ( $40 \times 10^6$ ) were incubated for 1 hr with 200 µCi  $^{51}\text{Cr}$  (Rotem Tassiot, Dimona, Israel) in 200 µl saline, 400 µl FCS and 300 µl complete medium. Following incubation, cells were washed 3 times (300 g, for 10 min) and adjusted to the desired concentration in complete media.

**Assessment of cytotoxicity.** Blood or spleen leukocytes were co-cultured in duplicate in microtiter plates (200 µl/well) with  $^{51}\text{Cr}$ -labeled YAC-1 target cells in 4 E:T ratios decreasing by 2-fold

from 50:1 in the blood mononuclear assay and from 100:1 in the spleen leukocyte assay. Target cells were incubated in either complete medium or 1 N HCl to determine spontaneous and maximal release of  $^{51}\text{Cr}$ , respectively. Plates were centrifuged at 200 g for 8 min prior to incubation and again before harvesting of 100 µl of the supernatant to determine  $^{51}\text{Cr}$ -release using a gamma-counter. The percent specific lysis was calculated for each E:T ratio using the formula [(experimental release – spontaneous release)/(maximum release – spontaneous release)]  $\times$  100.

### Anti-NKR-P1

The anti-NKR-P1 monoclonal antibody (anti-rat NKR-P1A; PharMingen, San Diego, CA), originally termed MAb 3.2.3, binds to a surface antigen (NKR-P1) expressed on fresh and IL-2-activated NK cells in rats and, to a much lesser degree, on polymorphonuclear (PMN) cells (Chambers *et al.*, 1992). *In vivo* treatment of rats with anti-NKR-P1 selectively depletes large granular lymphocyte (LGL)/NK cells and eliminates NK- and antibody-dependent, non-MHC-restricted cell cytotoxicity. T-cell function and the percentage of T cells, monocytes and PMN cells were unaffected (Chambers *et al.*, 1989; van den Brink *et al.*, 1991). Conjugated with FITC, anti-NKR-P1 was used in FACS analysis to identify NK cells.

### Flow cytometry

An aliquot of 100 µl of blood was combined with 50 µl of PBS (supplemented with 2% FCS and 0.1%  $\text{NaN}_3$ ) and 0.1 µg FITC-conjugated anti-NKR-P1. Samples were kept in the dark at room temperature thereafter. Following a 15-min incubation period, 2 ml FACS lysis solution was added (Becton Dickinson, Mountain View, CA), and 10 min later, samples were centrifuged for 5 min at 500 g and the lysis solution was aspirated. Cells were washed again with 2 ml PBS (5-min centrifugation, 300 g) and resuspended in 300 µl of PBS for flow-cytometric analysis using a FACScan (Becton Dickinson).

The criterion for positive identification of spontaneously active LGL/NK cells was defined as being above a level of fluorescence intensity that distinguishes between brightly and dimly stained populations of NKR-P1-positive cells, as described previously by Chambers *et al.* (1989, 1992). These previous studies also demonstrated that NKR-P1 is expressed by 94% of blood LGL cells and that the NK cytolytic activity was completely contained in the NKR-P1 bright cell population. PMN leukocytes expressed low levels of NKR-P1 and categorized as dim cells, and macrophages and mast cells were negative (Chambers *et al.*, 1989). In our studies, bright cells were defined as showing above 150 relative fluorescence intensity units, a level that distinguished between the 2 non-overlapping populations of the dim and bright NKR-P1<sup>+</sup> cells. Non-specific binding was assessed using nonspecific IgG<sub>1</sub>, which consistently yielded 0% brightly stained cells.

### Whole-blood NKA assay

This 4-hr cytotoxicity procedure assesses anti-tumor NKA per milliliter of blood without prior purification of PBMCs (or the exclusion of any cell population). It reduces the time between blood withdrawal and assessment of cytotoxicity and lessens the potential interference with NK cell function. Our previous studies (Ben-Eliyahu *et al.*, 1996a; Page *et al.*, 1994) indicate that cytotoxicity in this assay depends on NK cells since their selective depletion nullified all specific killing.

Blood was drawn into a heparinized syringe containing 25 U/ml of blood. Exactly 1 ml of blood was washed once with PBS (diluted 1:4 vol/vol, centrifuged at 300 g for 10 min and supernatant aspirated to original blood volume) and twice with complete medium. For each of the 6 E:T ratios used, 100 µl of washed blood were placed into each well of a microtiter plate and 150 µl of  $^{51}\text{Cr}$ -labeled YAC-1 tumor cells in complete medium added on top of the blood. A concentration of  $1.6 \times 10^6$ /ml YAC-1 was used for the lowest E:T ratio (approximately 1:8 NK:YAC-1, depending on individual number of NK cells/ml of blood) and sequentially

diluted by 2 to produce higher E:T ratios (approx. 4:1 at the highest). Spontaneous (SP) and maximal (MAX) releases of  $^{51}\text{Cr}$  from target cells were determined by substituting blood with complete medium or 4% HCl, respectively. Plates were centrifuged at 500 g for 10 min to create a buffy coat layer of leukocytes and target cells on top of the red blood cells prior to a 4-hr incubation period. Following incubation, plates were again centrifuged and aliquots of 100  $\mu\text{l}$  of the supernatant were recovered from each well for assessment of radioactivity in a gamma-counter. SP and MAX releases of radioactivity from tumor cells were measured separately for each of the 6 tumor concentrations, and percent specific lysis was calculated for each E:T ratio using the formula  $[(0.8 \times X - \text{SP})/(\text{MAX} - \text{SP})] \times 100$ , where X is the experimental release. The X value is multiplied by 0.8 to correct for the reduction in the supernatant volume into which  $^{51}\text{Cr}$  is released. This reduction is caused by the presence of RBCs in the wells (MAX and SP are assessed in the absence of RBCs).

#### MADB106, CRNK-16 and C4047 tumor lines

MADB106 is a selected variant cell line obtained from a pulmonary metastasis of a mammary adenocarcinoma (MADB100) chemically induced in inbred F344 rats (Barlozzari *et al.*, 1985). The C4047 colon cancer and the CRNK-16 leukemia are also syngeneic in F344 rat. Following an i.v. injection, C4047 was reported to colonize the lungs (Yeager and Colacchio, 1991).

The CRNK-16 leukemia occurs spontaneously in aged F344 rats and is a major cause of natural death (Ward and Reynolds, 1983). All 3 cell lines were maintained in complete medium at 100% humidity, 5%  $\text{CO}_2$  at 37°C. MADB106 and C4047 cell lines were maintained in monolayer cultures and separated from the flask using 0.25% trypsin.

#### Radiolabeling of tumor cells and assessment of lung tumor retention

For assessment of lung tumor retention, DNA radiolabeling of tumor cells was accomplished by adding 0.4 mCi/ml of  $^{125}\text{I}$ -iododeoxyuridine ( $^{125}\text{IDUR}$ ) (ICN, Irvine, CA) to the growing cell culture 1 day before harvesting the cells for injection.

For tumor cell injection, rats were lightly anesthetized with halothane and  $4 \times 10^5/\text{kg}$   $^{125}\text{IDUR}$ -labeled MADB106 tumor cells in approximately 0.5 ml of PBS injected into the tail vein. Twenty-four hours later, rats were euthanized with halothane and their lungs removed and placed in a gamma-counter for assessment of radioactive content. The percentage of tumor cells retained was calculated as the ratio of radioactivity measured in the lungs to total radioactivity in the injected tumor cell suspension. Our previous studies have indicated that the levels of lung radioactivity reflect the numbers of viable tumor cells in the lungs (Ben-Eliyahu and Page, 1992).

#### Selective in vivo depletion of NK cells

Two days before tumor inoculation, approximately 1.5 mg/kg of anti-NKR-P1 were injected i.v. under light halothane anesthesia. Controls were injected with vehicle. In a previous study using the above-mentioned dose of the anti-NKR-P1, we demonstrated abolition of blood and splenic NK cytotoxicity and a 100-fold increase in lung retention and metastatic colonization of MADB106 tumor cells (Ben-Eliyahu and Page, 1992). In addition, we have used other MAbs (R73, W3/25, ED2), mouse serum and saline as controls for the administration of anti-NKR-P1 and have found no effects (Ben-Eliyahu and Page, 1992).

#### Counterbalancing, combining results and statistics

The time and order of spleen removal, blood withdrawal and tumor injection were counterbalanced across all groups in all experiments (*i.e.*, conducted in all groups in parallel). For each experiment, any one of these procedures was completed within less than 2 hr. In some studies, a large number of animals was needed; these studies were conducted over 2 or more sessions, and results were combined based on control levels from the different sessions.

For statistical analysis, ANOVA was conducted, and provided significant group differences existed, planned contrasts or Scheffe *post hoc* tests were used to identify specific differences. In 2 cases (experiments 1 and 3), ANOVA was not used because marked variances characterized the different groups, thereby violating an ANOVA assumption. Instead, we used *t*-tests to compare the control group with each of the stressed groups, employing alpha correction for multiple *t*-tests. Results of the study with CRNK-16 leukemia were analyzed by the SURVIVAL Kaplan-Meier procedure. An alpha of 0.05 was set for all experiments.

## PROCEDURES AND RESULTS

### Experiment 1: Time course of effects of surgical stress on NKA and MADB106 tumor metastasis

**Splenic and blood mononuclear NKA.** The spleen and blood are 2 major immune compartments in which NK cells are found and between which NK cells migrate (Schedlowski *et al.*, 1996). Whereas in human studies only blood can be used as a source of NK cells, animal studies have the advantage of enabling the use of either spleen or blood cells. In this experiment, we assessed NKA in both compartments to increase the generalizability of the findings. In most rats, both the spleen and blood were taken simultaneously; in some rats, due to technical limitations, only one could be taken.

Splenic and blood mononuclear NKA was assessed in 3 replicates, each between 8:00 and 10:00 A.M. Rats underwent surgery at either 5, 12 or 24 hr or 7 days prior to spleen removal ( $n = 17, 6, 17$  and  $14$ , respectively) and blood drawing (right ventricular puncture,  $n = 13, 7, 13$  and  $13$ , respectively). Control rats ( $n = 40$  and  $30$  for spleen and blood, respectively) were evenly distributed across the 3 replicates.

The number of lytic units per  $10^7$  cells (at 25% specific killing) was calculated for each sample assessed for NKA (Pollock *et al.*, 1990). NKA in both blood and spleen was reduced at 5, 12 and 24 hr after surgery but not at 7 days (Fig. 1a, b). ANOVA indicated significant group differences in blood mononuclear NKA [ $F(4,71) = 3.441, p < .05$ ] and in splenic NKA [ $F(4,89) = 3.337, p < .05$ ]. *Post hoc* analysis indicated significant differences between control levels and 5 and 24 hr levels in the blood and between control levels and 12 and 24 hr levels in the spleen.

**Lung tumor cell retention of MADB106.** Unlike the above *in vitro* assessment of NKA, which reflects the *in vivo* condition at the one time point at which blood and spleen were taken, the lung tumor retention procedure cumulatively assesses host resistance to MADB106 metastasis over a 24-hr period. Thus, we used rats that underwent surgery 5 or 24 hr or 1 week prior to tumor inoculation and skipped the 12-hr point used in the previous study.

Rats either served as control ( $n = 12$ ) or underwent abdominal surgery at 5 or 24 hr or 1 week ( $n = 7, 12$  and  $10$ , respectively) prior to the injection of radiolabeled MADB106 tumor cells. Rats were killed 24 hr after tumor inoculation for assessment of lung tumor retention.

A significant increase in lung tumor retention was evident in rats which had undergone surgery at either 5 or 24 hr before tumor inoculation ( $t = 15.807, p < 0.005; t = 7.174, p < .02$ , respectively) but not 7 days prior to tumor inoculation (Fig. 1c). A separate comparison between the control group and each stressed group was conducted, using alpha correction for multiple *t*-tests, because the marked variances characterizing the different groups violated an ANOVA assumption.

### Experiment 2: Time course of effects of swim stress on number and activity levels of blood NK cells and on tumor development

**Number of WBC and NK cells per milliliter of blood.** Various stress hormones are known to affect the number of NK cells per milliliter of blood and the percentage of NK cells within the blood leukocyte/mononuclear cell population (Benschop *et al.*, 1996; Dhabhar *et al.*, 1995). Therefore, in this study, we first assessed the

number of NK cells per milliliter of blood at different time points following stress.

Rats either served as control (n = 24) or underwent swim stress at either 40 min or 1, 3, 6, 12 or 18 hr before 1 ml of blood was

drawn by cardiac puncture under light halothane anesthesia into syringes containing 20 U heparin (n = 4, 9, 18, 18, 8 and 12, respectively). The time of blood draw was counterbalanced across all groups, between 12:00 and 2 P.M. (middle of the dark phase). The number of WBCs per milliliter of blood was assessed using a Coulter (Hialeah, FL) counter, and the percentage of LGL/NK cells (NKR-P1-positive bright cells) was assessed using flow cytometry, as described above.

The results are shown in Figure 2. The number of WBCs per milliliter blood significantly decreased at 40 min and 1 hr after stress, returned to normal at 3 and 6 hr after stress and was significantly higher than normal at 12 and 18 hr after stress. Conducting a flow-cytometric analysis, a cut-off of 150 relative fluorescence intensity units was set to distinguish between the NKR-P1<sup>+</sup> bright cells (LGL/NK cells) and the non-overlapping populations of NKR-P1<sup>+</sup> dim cells (PMN cells). This analysis indicated that the percent of LGL/NK cells within the population of WBCs significantly increased at 40 min and 1 hr, significantly decreased at 3 and 6 hr and returned to normal at 12 and 18 hr after stress. The number of LGL/NK cells per milliliter of blood (% of LGL/NK × WBC/ml) was significantly higher than control at 40 min, returned to control levels at 1 hr, was significantly lower at 3 hr, was not different from control at 6 and 12 hr and increased slightly but significantly at 18 hr post-stress.

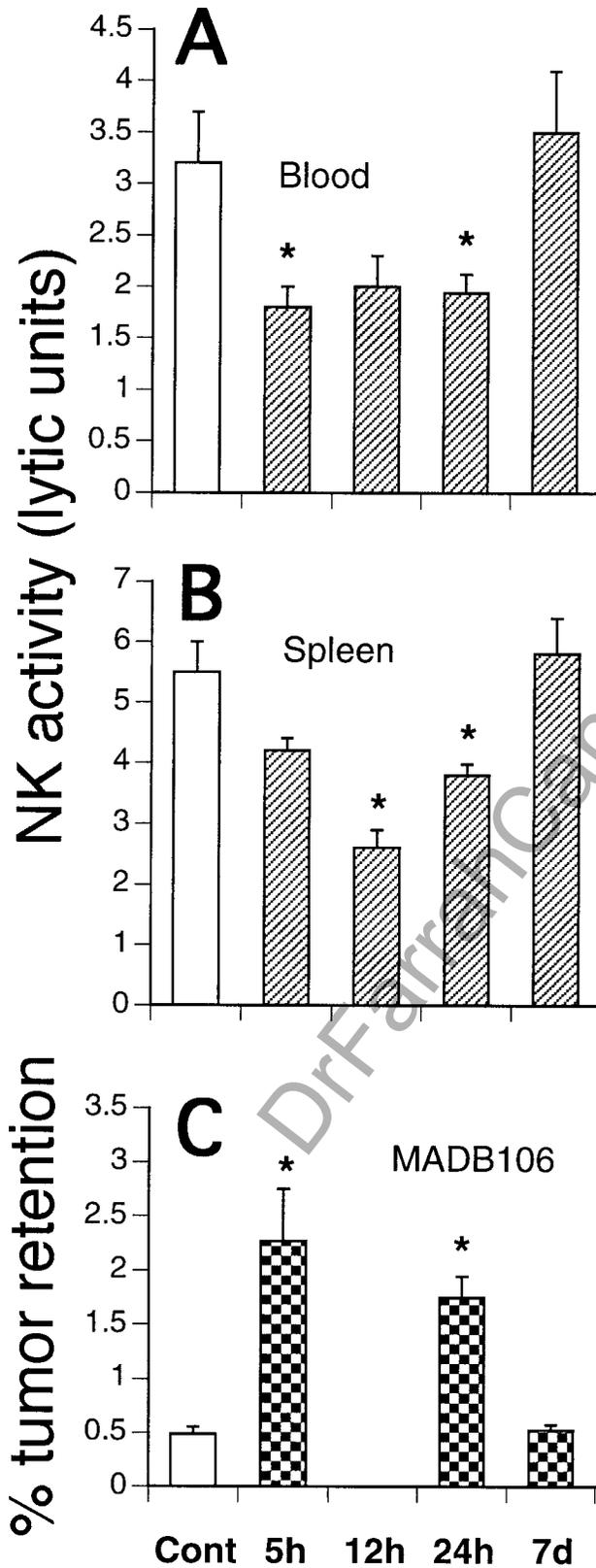
**Blood levels of NKA.** Based on the results of our previous study, blood was drawn and NKA assessed at times at which the number of NK cells per milliliter of blood was similar in control and stressed animals. At these time points, alteration in NK cytotoxicity per milliliter of blood also reflects alteration in NK cytotoxicity per NK cell.

NK cytotoxicity per milliliter of blood was assessed using the whole-blood procedure described above. Rats either served as controls (n = 15) or underwent swim stress 1 or 12 hr before blood was drawn by cardiac puncture under light halothane anesthesia (n = 14 and 13, respectively).

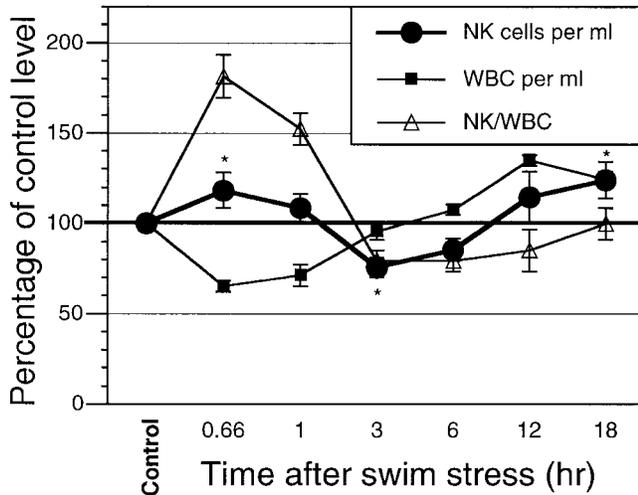
The number of lytic units per milliliter of blood (at 30% specific killing) was calculated for each sample assessed for NKA (Pollock *et al.*, 1990). Swim stress significantly suppressed NKA per milliliter of blood at 1 hr post-stress but not at 12 hr post-stress (Fig. 2a). ANOVA revealed significant group differences [ $F(2,39) = 3.44, p < 0.05$ ], and planned contrasts indicated a significant suppression of NKA only in rats stressed 1 hr prior to blood withdrawal.

Because at the time points at which blood was drawn the number of LGL/NK cells per milliliter of blood was similar in stressed and control animals, the suppressed NK cytotoxicity per milliliter of blood at 1 hr post-stress and the absence of an effect at 12 hr hold for NK cytotoxicity per LGL/NK cell as well.

**Lung tumor cell retention of MADB106 and C4047 tumors.** In this experiment, we compared the effects of swim stress on lung tumor retention using the NK-sensitive MADB106 line with the effects using the NK-insensitive C4047 line. In addition, a more detailed time course of the effects of swim stress on the NK-sensitive MADB106 tumor was conducted.



**FIGURE 1** – Time course of the effects of surgery on NK activity in blood (a) and spleen (b) (both in lytic units) and on MADB106 lung tumor retention (c). Rats served as controls (Cont) or underwent surgery at either 5, 12 or 24 hr or 7 days before blood and spleen were taken, or at either 5 or 24 hr or 7 days before MADB106 tumor cells were inoculated for the assessment of 24-hr lung tumor retention (rats were not inoculated with the tumor at 12 hr after surgery because the 24-hr period starting at this point is covered by the 5- and 24-hr groups). Significant suppression of blood and splenic NK activity was evident up to 24 hr post-surgery but not at 7 days. Correspondingly, a significant increase in tumor retention was evident if tumor cells were injected up to 24 hr post-surgery but not at 7 days. Error bars represent SEM. \*Significant difference from the respective control group.



**FIGURE 2** – Time course of the effects of swim stress on the number (mean  $\pm$  SEM) of NK cells (NKR-P1<sup>bright</sup>) and WBCs per milliliter of blood and on the percentage of NK cells within the WBC population (NK/WBC). Data are presented as percentages of control:  $22.5 \times 10^4$  ( $\pm 0.2$ ) NK cells/ml blood,  $6.6 \times 10^6$  ( $\pm 0.2$ ) WBCs/ml blood and 3.5% ( $\pm 0.1$ ) NK/WBCs. \*Significant difference only for number of NK cells/ml blood. Significant differences in respect to WBCs and NK/WBCs are noted in “Results”.

Control and swim-stressed rats were injected with either MADB106 or C4047 cells 1 hr after stress, and lung tumor retention was assessed 24 hr after stress ( $n = 6$  or  $7$  in each of these 4 groups). Since experiment 2 indicated that NKA is suppressed only during the first hours after swim stress, the above 24-hr interval was divided and the effects of swim stress on lung tumor retention of the NK-sensitive MADB106 line were assessed between 1 and 8 hr and between 8 and 24 hr after stress: one control and one stressed group were injected with MADB106 at 1 hr after stress and killed at 8 hr after stress (first period); another control and another stressed group of rats were injected at 8 hr after stress and killed at 24 hr after stress (second period) ( $n = 6$  or  $7$  in each of these 4 groups). All rats from the above 8 groups were injected between 12:00 and 2:00 P.M. (middle of the dark phase).

When tumor was injected 1 hr after stress and lungs removed at 24 hr, swim stress significantly increased lung tumor retention of the NK-sensitive MADB106 line but not of the NK-insensitive C4047 line, though similar levels of lung tumor retention were observed in control groups injected with the 2 tumor lines (Fig. 3). ANOVA indicated significant group differences [ $F(3,20) = 18.8$ ,  $p < 0.0001$ ], and *post hoc* Scheffe comparisons indicated that only the stressed group injected with MADB106 cells had higher tumor retention than its respective control group. The tumor-enhancing effect of swim stress on the NK-sensitive MADB106 tumor occurred during the first period (1 to 8 hr after stress) but not during the second period (8 to 24 hr after stress) (Fig. 2b). ANOVA indicated significant group differences [ $F(3,22) = 15.1$ ,  $p < 0.0001$ ], and *post hoc* Scheffe comparisons indicated that only the stressed group assessed for tumor retention during the first period had significantly higher levels than its respective control group.

**Development of NK-sensitive CRNK-16 leukemia.** Forty-six rats were injected i.v. with  $10^4$  CRNK-16 leukemia cells between 10:00 and 11:00 A.M. Eighteen of these rats served as controls and 28 were exposed to swim stress 1 hr prior to tumor injection. Mortality and morbidity were assessed daily during the following 3 months. Rats that lost more than 40% of their body weight or became paralyzed were euthanized and included in the reported mortality rate.

Swim stress significantly increased mortality rate in animals inoculated with the CRNK-16 leukemia line (Kaplan-Meier test,

Tarone-Ware = 5.14, 1 df,  $p < 0.05$ ). As seen in Figure 4, the first animal died on the 28th day post-inoculation and similar levels of mortality were observed between the 2 groups up to day 31 (30%). Thereafter, the stressed group showed a higher mortality rate: by day 43, all stressed rats had died, whereas 33% of the control rats had survived. By day 90, when the experiment was terminated, no survivor had shown any sign of morbidity.

#### Experiment 3: Effects of swim and surgical stress on MADB106 lung tumor retention in normal and LGL/NK-depleted rats

To further study the role of LGL/NK cells in mediating the effects of stress on lung tumor retention of the NK-sensitive MADB106 tumor, we compared the effects of swim and surgical stress in normal rats to their effects in rats selectively depleted of LGL/NK cells. If LGL/NK cells are necessary to mediate the effects of stress, then these effects should not be evident in LGL/NK-depleted rats. If, however, factors other than LGL/NK cells are sufficient to mediate the effects of stress (e.g., alteration in vascular permeability to tumor cells), then these effects should still be evident in LGL/NK-depleted rats.

Two days before tumor injection, half the rats were injected with anti-NKR-P1 to selectively deplete LGL/NK cells, whereas the other half received a control injection (see “Material and Methods” for a detailed description of the depletion procedure and control injections). Rats from each of these 2 groups were then assigned to one of 3 subgroups ( $n = 6$  in each group): swim stress, surgical stress or non-stressed control. Lung tumor retention was assessed 24 hr after the inoculation of radiolabeled MADB106 tumor cells.

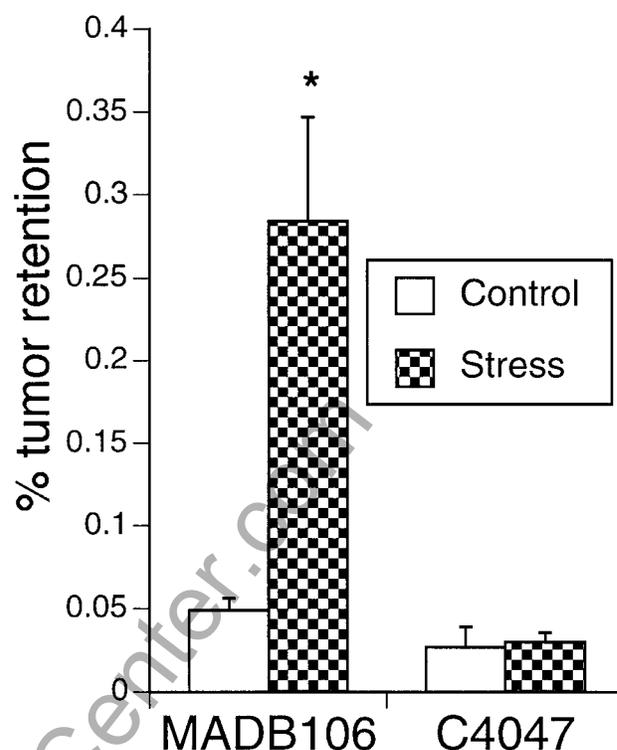
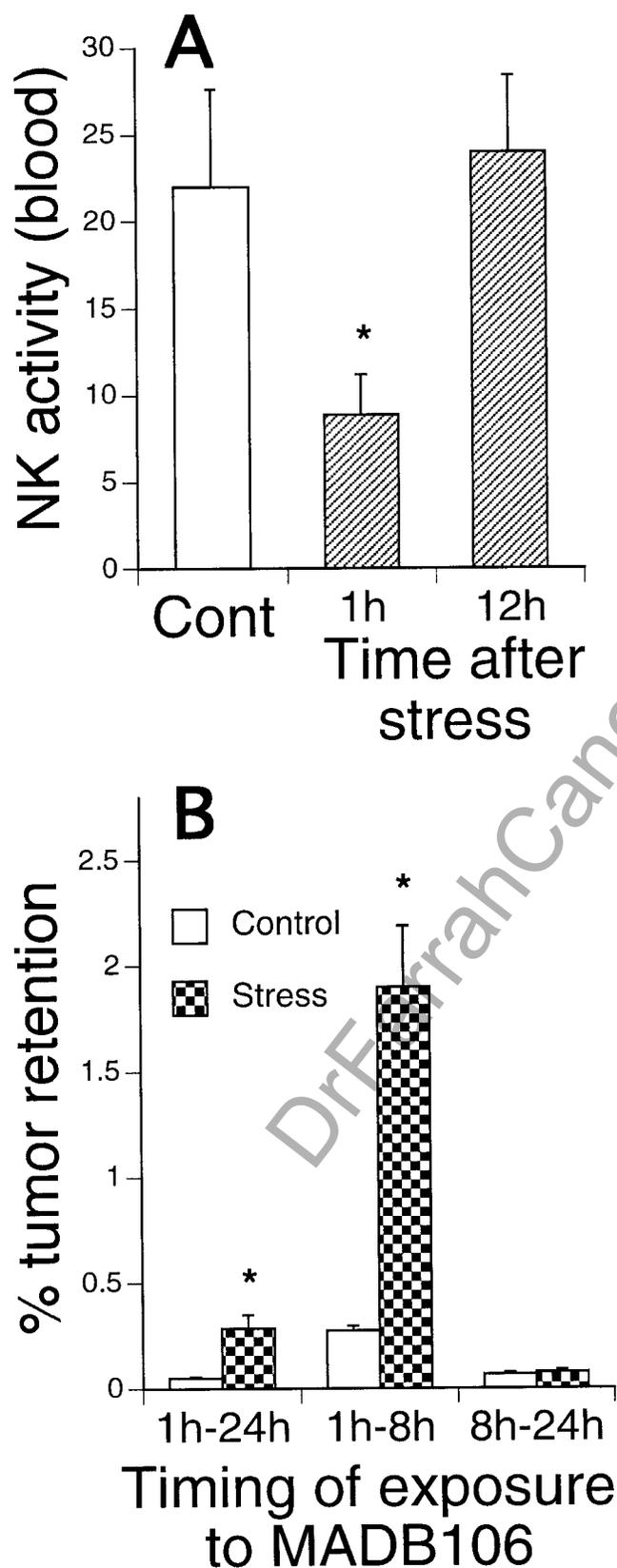
Depletion of LGL/NK cells caused an approximately 60-fold increase in lung retention of tumor cells (LGL/NK-depleted, non-stressed controls vs. normal non-stressed controls), demonstrating the high sensitivity of this measure to LGL/NK cell activity (Fig. 5). Surgery caused a significant, 50% to 70%, increase in tumor retention in both normal ( $t_{10} = 3.2$ ,  $p < .05$ ) and LGL/NK-depleted ( $t_{10} = 7.4$ ,  $p < .05$ ) rats, demonstrating a non-LGL/NK-mediated effect in LGL/NK-depleted rats. Swim stress, however, caused a significant, 320%, increase in tumor retention in normal rats ( $t_{10} = 6.8$ ,  $p < .05$ ) but had no effect in LGL/NK-depleted rats ( $t_{10} = 0.97$ ,  $p = 0.36$ ) (Fig. 5), indicating that the effect of swim stress is mediated by LGL/NK cells. The fact that swim stress did not affect lung tumor retention in LGL/NK-depleted rats cannot be attributed to a ceiling effect because surgery did cause a significant increase in tumor retention in depleted rats. Surgery should not be considered a more powerful stimulus than swim stress under these circumstances; indeed, swim stress had a greater effect on lung tumor retention than did surgery in normal rats. Separate *t*-tests were employed to compare each stressed group to the control group because marked variances characterized the NK-depleted and normal groups, thereby violating an ANOVA assumption.

## DISCUSSION

Our results indicate that host resistance to tumor development is compromised by swim and surgical stress; i.e., a higher mortality rate from CRNK-16 leukemia and increased lung retention of MADB106 tumor cells were observed following stress. Our previous studies indicated that tumor development is also increased by social confrontation stress (Stefanski and Ben Eliyahu, 1996) or by the administration of adrenaline or  $\beta$ -adrenergic agonists (Ben-Eliyahu, 1998; Shakhar and Ben-Eliyahu, 1998). These findings together with reports on the effects of stress in other experimental tumor models (Yirmiya *et al.*, 1991) clearly demonstrate the deleterious effects of acute stress on host resistance to tumor development.

The main focus of our study was to assess the specific role of NK cells in mediating the effects of stress on tumor development. To this end, it is important to understand the role of NK cells in controlling the development of each of the 3 syngeneic tumor lines used. Two lines are controlled by NK cells (CRNK-16 and the MADB106) and one is not (colon C404). Specifically, CRNK-16

leukemia is readily killed *in vitro* by activated NK cells (Reynolds, 1985; Ward and Reynolds, 1983), and ample evidence has indicated the *in vivo* sensitivity of MADB106 to LGL/NK cell activity.



**FIGURE 4**—Effects of swim stress on lung tumor retention of the NK-sensitive MADB106 line and the NK-insensitive C4047 line. Rats were inoculated with either tumor cell line 1 hr after stress and lungs were removed 24 hr later. Although similar levels of tumor retention were evident in the control group, stress significantly increased tumor retention in the MADB106, but not the C4047, group. Error bars represent SEM. \*Significant difference from the respective control group.

MADB106 cells metastasize only to the lungs (Barlozzari *et al.*, 1985; Ben-Eliyahu and Page, 1992). Earlier research has shown a significant increase in lung tumor retention and a 10-fold increase in the number of surface lung metastases when NKA was reduced with anti-asialo GM<sub>1</sub>. These effects were prevented by adoptive transfer of relatively few purified LGL/NK cells, but not T cells, prior to tumor injection (Barlozzari *et al.*, 1983, 1985). Using the anti-NKR-P1 MAb, which selectively depletes LGL/NK cells and eliminates NKA without affecting other immune functions (Ben-Eliyahu and Page, 1992; Chambers *et al.*, 1989, 1992; van den Brink *et al.*, 1991), more than a 100-fold increase in MADB106 lung tumor retention and an equivalent increase in the number of lung metastases were found (Ben-Eliyahu and Page, 1992; Ben-Eliyahu *et al.*, 1991, 1996a). At least 70% of MADB106 cells were

**FIGURE 3**—Time course of the effects of swim stress on blood NK activity (a) and on MADB106 lung tumor retention (b). (a) Rats served as controls (Cont) or were exposed to stress, and blood was drawn at either 1 or 12 hr after stress, the times at which the number of NK cells per milliliter of blood was similar to controls (Fig. 2). NK activity is presented as lytic units. (b) Control and stressed rats were inoculated with the NK-sensitive MADB106 tumor 1 hr after stress, with lungs removed at either 24 hr (1h-24h) or 8 hr (1h-8h) after stress. Other control and stressed rats were inoculated at 8 hr after stress with lungs removed at 24 hr after stress (8h-24h). NK activity per milliliter of blood (and per NK cell) was suppressed at 1 hr, but not at 12 hr, after stress (a). Correspondingly, stress increased tumor retention when exposure to MADB106 began 1 hr, but not 8 hr, after stress (b). Error bars represent SEM. \*Significant difference from the respective control group.

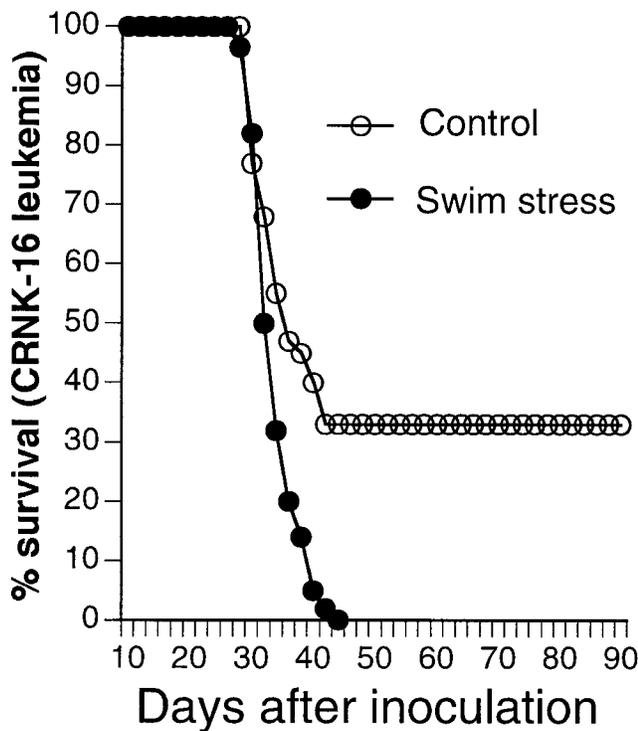


FIGURE 5 – Effects of swim stress on survival following i.v. inoculation with the CRNK-16 leukemia line. Rats either served as controls or were exposed to stress 1 hr before tumor inoculation. Stress significantly increased mortality rate.

destroyed *in vivo* by LGL/NK-dependent activity within 5 hr of inoculation, a destruction rate that paralleled the clearance of tumor cells from the lungs. In contrast, administration of anti-NKR-PI 1 day after tumor inoculation, when tumor cells have already extravasated and seeded in the lungs, had no effect on the number of lung metastases (Ben-Eliyahu and Page, 1992). Finally, increased NKA (induced by poly I:C) was associated with decreased lung tumor retention and metastatic colonization (Ben-Eliyahu and Page, 1992), and various conditions under which NKA was reduced (*e.g.*, ethanol consumption and high levels of morphine), also increased lung tumor retention and metastatic colonization of the MADB106 tumor (Ben-Eliyahu *et al.*, 1996b).

Together, the above results indicate that (i) lung tumor retention is an early indicator of metastatic colonization of the MADB106 tumor, (ii) both lung tumor retention and metastatic colonization sensitively reflect *in vivo* activity levels of NK cells and (iii) MADB106 tumor cells are sensitive to NKA predominantly during the first 24 hr following i.v. inoculation. Thus, the assessment of a 24-hr lung tumor retention focuses on the impact of NK cells on the metastatic process and reduces the impact of other factors which may affect the growth of metastases at later stages.

In contrast with MADB106, lung tumor retention of the C4047 tumor line is unaffected by LGL/NK cell activity in that selective depletion of LGL/NK cells had no effect on C4047 lung tumor cell retention (Ben-Eliyahu *et al.*, 1996b). This tumor line metastasizes to the lungs following i.v. inoculation (Yeager and Colacchio, 1991), and its baseline lung retention levels were found, in both the current study and a previous one (Ben-Eliyahu *et al.*, 1996b), to be similar to those of MADB106.

Considering the above and addressing the role of NK cells in mediating the effects of stress on tumor development, our finding that swim stress increased the development of the NK-sensitive CRNK leukemia and MADB106 metastasis but not the NK-

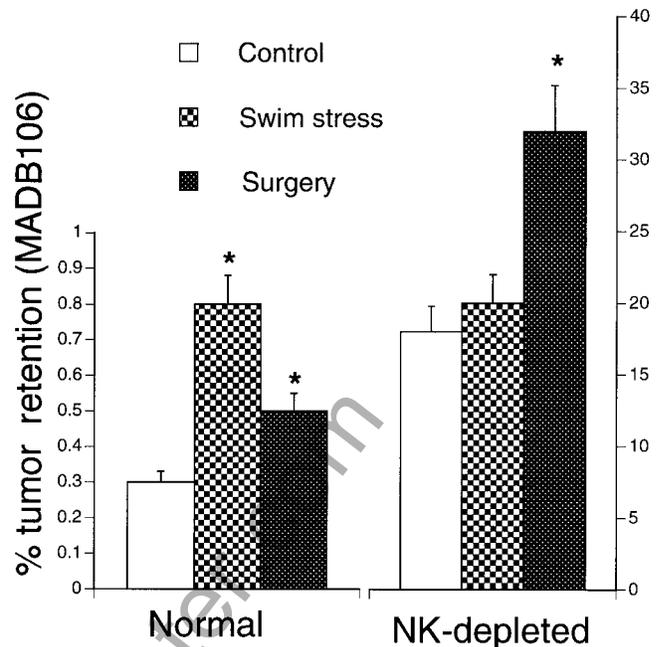


FIGURE 6 – Effects of swim stress and surgery on lung tumor retention of the NK-sensitive MADB106 line in normal and in LGL/NK-depleted rats. Depletion caused a 60-fold increase in tumor retention in control rats (note different scales). Swim stress caused a significant 3-fold increase in tumor retention in normal rats but had no effect on tumor retention in LGL/NK-depleted rats, suggesting that the effect of swim stress is mediated primarily by LGL/NK cells. However, surgery caused a similar effect in normal and LGL/NK-depleted rats (a significant 150% to 170% increase), indicating that surgery can increase tumor retention *via* mechanisms other than LGL/NK cells and negating the possibility of a ceiling effect in LGL/NK-depleted rats. Error bars represent SEM. \*Significant difference from the respective control group.

insensitive C4047 metastasis suggest that in the current study NK cells mediated the effects of stress on tumor development.

The second line of evidence in support of this suggestion is the similarity between the time course of the NK-suppressive effects of stress and the time course of the metastasis-enhancing effects of stress. This similarity was found using both the swim and the surgical stress paradigms, though the course of the effects of these 2 stressors was markedly different. Specifically, throughout the first day after surgery (at 5, 12 and 24 hr) but not at 7 days after surgery a significant suppression of NKA was observed in spleen and blood mononuclear cells. Similarly, surgery increased lung tumor retention if tumor cells were injected at either 5 or 24 hr after surgery but not at 7 days after surgery. Blood NKA was suppressed at 1, but not at 12, hr after swim stress. Similarly, swim stress increased MADB106 metastasis for a few hours only: increased lung tumor retention was evident when tumor cells were injected at 1 hr post-stress, but no effect was evident when tumor cells were injected at 8 hr post-stress. The reduction in NKA 1 hr following swim stress is attributable to the decreased activity per LGL/NK cell as the number of LGL/NK cells within the cell population tested for NKA at this time point resembled that of control levels. Our previous studies also demonstrated suppression of splenic and blood mononuclear NKA at 1 hr post-swim stress when NK cytotoxicity was assessed *in vitro* against the syngeneic MADB106 tumor line, rather than the standard xenogeneic YAC-1 tumor line used in the current study (Ben-Eliyahu *et al.*, 1991). Thus, the suppressive effects of swim stress on NKA are likely to be independent of cell trafficking, generalizable to immune compartments other than the blood or spleen and likely to underlie the

increased lung tumor retention of the NK-sensitive MADB106 tumor.

The evidence discussed to this point clearly suggests a role for NK cells in mediating the effects of stress on tumor development. Nevertheless, as all of this evidence is circumstantial, it could be argued that other factors may underlie the effects of stress on tumor development. More direct evidence that LGL/NK cells mediate the effects of stress on tumor development is found in the study employing LGL/NK-depleted rats. Whereas in normal rats both swim and surgical stress caused a marked and significant increase in MADB106 lung tumor retention, in LGL/NK-depleted rats swim stress did not increase tumor retention, though surgical stress did. These findings indicate that LGL/NK cells are a necessary mediator of the effects of swim stress on MADB106 metastasis. In addition, these findings suggest that the effects of surgical stress were not mediated exclusively by LGL/NK cells; other mechanisms participated in mediating the effects of surgical stress.

It might be argued that the lack of swim stress effects in LGL/NK-depleted rats resulted from a ceiling effect. However surgical stress significantly and markedly increased tumor retention in LGL/NK-depleted rats, in which swim stress had no effect. Surgery should not be considered a more severe stressor in this context as it produced a smaller effect than swim stress in normal rats. In other studies (data not shown), the effects of surgical stress were abrogated by depletion of LGL/NK cells. Thus, certain unknown conditions of the host, such as immune activation, may determine whether factors other than NK cells play a role in mediating surgery-induced increased metastasis.

Together, these 3 converging lines of evidence indicate that suppression of NKA induced by stress or by surgery is sufficient to promote tumor development. Whereas some stressors (e.g., forced swimming) promote tumor development predominantly via suppression of NKA, other stressors (e.g., surgery) enhance tumor development via multiple mechanisms which most likely include the suppression of NKA.

The neuroendocrine mechanisms mediating the effects of stress on immunity have been the focus of many studies in which various stress hormones were implicated (Ader *et al.*, 1991). We have reported that the systemic administration of an adrenergic  $\beta$  agonist suppressed NKA in the rat and, consequently, reduced host resistance to tumor metastasis (Shakhar and Ben-Eliyahu, 1998).

Various interventions interfering with sympathetic nervous system functioning were found to be effective at blocking the NK-suppressive and/or tumor-enhancing effects of stress and of other manipulations (Ben-Eliyahu, 1998; Brenner *et al.*, 1992; Irwin *et al.*, 1990). Finally, human studies have also implicated the peripheral release of catecholamines in modulating NKA under various conditions (Hellstrand and Hermodsson, 1989; Moyna *et al.*, 1996; Schedlowski *et al.*, 1996; Tonnesen *et al.*, 1993).

In humans, surgical procedures (Andersen *et al.*, 1998; Pollock *et al.*, 1992) as well as other major stressors (Cohen and Herbert, 1996) have been reported to suppress NKA and other immune functions for several days. The biological significance of such immune suppression is suggested by the well-established relationship between surgical procedures and the outbreak of dormant infectious diseases known to be under immune surveillance, such as tuberculosis, herpes or cytomegalovirus (Glaser *et al.*, 1987; Holzheimer *et al.*, 1997). Whether such immune-suppressive effects of surgery also impact tumor development in the clinical setting remains unknown. However, many patients undergo surgery for the excision of a neoplasm with metastatic potential at the time of surgery, as evidenced by the presence of positive lymph nodes or the detection of metastases. If our findings in rats can be generalized to such clinical settings, then suppression of NKA or other immune functions under such circumstances may increase the susceptibility to tumor metastasis during or shortly after surgery.

In conclusion, based on the findings of this and other studies, we suggest that under specific circumstances various stressors, including surgical stress, can enhance metastasis by suppressing the cytotoxic activity of NK cells. Because surgical procedures are life-saving and, thus, cannot be withheld, prophylactic measures against their potential immune-suppressive and metastasis-enhancing effects should be considered.

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