Acta Biologica Hungarica 54 (2), pp. 167–176 (2003)

RESTRAINT STRESS AND ANTI-TUMOR IMMUNE RESPONSE IN MICE*

V. Posevitz,[1] C. Vizler,[1**] S. Benyhe,[1] E. Duda[1,2] and Anna Borsodi[1]

[1] Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary
[2] Institute of Medical Microbiology, Albert Szent-Györgyi Medical and Pharmaceutical Center, Szeged University, Szeged, Hungary

(Received: April 2, 2003; accepted: April 24, 2003)

Psychological stress modulates the immune system through the hypothalamic-pituitary-adrenal axis, the sympatho-adrenomedullary axis and the opioid system. According to literature data, restraint stress increases the immune cell apoptosis, decreases the spleen and thymus cell content, the natural killer (NK) activity in the spleen, and it compromises the anti-tumor immune response in mice. We immobilized mice in two consecutive nights, and then determined the cell number, apoptosis, NK cell content, NK activity and the level of cytokine mRNAs (TNF-β, TNF-α, IL-4, IL-5, IL-1α, IFN-γ, IL-2, IL-6, IL-1β and IL-3) in the thymus and spleen. No consistent changes were detected in any of the immune parameters either in C57Bl/6 or in DBA/2 mice. Stressed or control B6 mice were injected with B16 melanoma cells immediately after the immobilization or one week later. No significant differences were found in the growth of primary tumors and lung metastases in stressed and control animals. Taken together, our mice, kept in a general-purpose non-SPF animal house, seemed to be refractory to the stress-induced immunomodulation. Our interpretation is that stress-induced immunomodulation can occur only in mice isolated from any background stressors, or rather natural stimuli, throughout their life.

Keywords: Psychological stress – apoptosis – NK cells – tumor immunity – opioids

INTRODUCTION

Psychological stress may possess immunomodulating effects both in humans and in rodents [30]. Beside the HPA and SAM axis, the opioid system is implicated as a mediator of the bi-directional interaction between the immune system and the nervous system [23, 25]. The original aim of our project was studying the role of different opioid agonists and receptor subtypes in the stress-induced immunomodulation. This line of research could lead to development of new therapeutical modalities for preventing the adverse effects of psychological stress, and it might also result in development of new opioid drugs, exempt from the immunosuppressive side effects of the opioids used in the clinical practice today.

* Dedicated to Professor Maria Wollemann on the occasion of her 80th birthday.

** Corresponding author; e-mail: vizler@nucleus.szbk.u-szeged.hu

The most frequently used stress model in rodents is immobilization (restraint) stress. Restraint stress has been reported to change the distribution of immune cells, resulting in decreased cell numbers in the thymus and spleen, decreased peripheral natural killer (NK) cell number and NK cell lytic activity, and increased lymphocyte number and cellular immunoreactivity in the skin [7–9, 20]. Stress-induced apoptotic death of splenocytes and thymocytes has also been described in these models. Restraint stress was also shown to suppress the antigen-specific humoral immune response in mice [12, 22]. In accordance with these results, psychological stress was reported to compromise the anti-viral immune response [2, 3]. Enhanced tumor susceptibility, attributed to compromised tumor-specific cytotoxic T lymphocyte (CTL) and NK response, has also been described [14, 16, 17].

In the first step of our project, we attempted establishing a mouse model of stress-induced immunomodulation. Mice were immobilized in plastic tubes for 2 × 12 hr in two consecutive nights – this protocol has been used in some of the most important publications of the field, which described a μ opioid receptor-dependent increase of CD95 expression leading to increased rate of lymphocyte apoptosis [27, 31]. In our experiments, cell numbers of the spleen and thymus, the percentage and lytic activity of splenic NK cells, splenocyte and thymocyte apoptosis, furthermore, TNF-α and IL-6 production of LPS stimulated spleen cells of stressed and control mice were compared. The levels of cytokine mRNAs (lymphotoxin α, TNF-α, IL-4, IL-5, IL-1α, IFN-γ, IL-2, IL-6, IL-1β and IL-3) in the thymus and spleen of stressed and control mice were quantified by ribonuclease protection assays.

In another series of experiments, stressed or control B6 mice were injected with syngeneic B16 melanoma cells immediately after the immobilization or one week later, then the growth of primary tumors and lung metastasis formation were quantified. This tumor model was chosen because the MHC-I_{low} B16 melanoma cells are known to be the sensitive to NK cell lysis, while, being MHC-I positive and having tumor-specific antigens, they are also potential targets of tumor-specific cytotoxic T lymphocytes (CTL) [19, 28].

MATERIALS AND METHODS

Mice and animal house conditions

C57Bl/6 (B6) and DBA/2 mice, obtained originally from Charles River (Margate, Kent, UK), were bred in our conventional animal house; they received Purina mouse food pellets and tap water *ad libitum*. Generally 7 animals of the same age were kept in non-enriched opaque plastic cages of 13 × 40 cm. The personnel of the animal house cleaned the boxes twice a week at regular intervals. The animal house was lit by natural light through windows, so the animals were exposed to the natural seasonal changes of the light-dark cycle. Our experiments were performed throughout the year; no difference was observed between the outcomes of the experiments performed in different seasons. The animal house was situated in a separated floor of a

research institute; it was relatively quiet and calm, but it was not completely isolated from the background noises of a typical research institute. In separate rooms of the animal house other animal species (rabbits and rats) were also kept.

Stress protocols

Three- to four-month old mice were used throughout the experiments. The stressed mice were put in horizontal 50 ml Falcon PVC centrifuge tubes with ventilation holes. Immobilization was performed from 9 p.m. to 9 a.m., i.e., in the active period of the day for mice, on two consecutive days. The *in vitro* experiments were performed immediately after the second immobilization. Tumor cell injections were performed either immediately after the immobilization, or one week later.

Beside the above protocol, a few experiments were performed using different stress protocols. In these experiments, mice were immobilized for varying time intervals: 2 hr, 12 hr, 24 hr. In another series of experiments, social confrontation was induced by putting adult male mice of different strain in common cages overnight. In a few experiments, spatial disorientation was induced by placing the cages of experimental animals on a shaker (30 horizontal tilts of 10°/min) overnight.

All animal experiments were performed in conformity with institutional and international animal welfare regulations.

In vitro experiments

Spleen and thymus cell suspensions were prepared in tissue culture medium (DMEM with 5% FCS) with Falcon cell strainers (BD Biosciences, Bedford, MA) and glass syringe plungers, then the contaminating red blood cells were lysed with a 5 min incubation in ACK red blood cell lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) at room temperature. Cell numbers were determined by counting the cell suspensions in a hemocytometer.

The NK cell content of the spleen was determined by flow cytofluorometric analysis performed after staining with FITC labeled anti-NK cell monoclonal antibody (clone DX5, BD Biosciences).

NK activity was measured by a flow cytometric method. Briefly, different numbers of effector spleen cells (E/T ratio = 1/10, 1/30, 1/100) or, alternatively, different fractions of spleen cells (1/10, 1/30 or 1/100 spleen) were added to 10^5 MitoTracker Green FM (Molecular Probes, Eugene, OR) labeled NK target cells (HeLa or K562 cells) in a volume of 0.5 ml. After 2.5 hr incubation, cells were stained with propidium iodide, and then live and dead target and effector cells were analyzed by a Becton Dickinson FACSCalibur flow cytofluorimeter, as it is described [26].

The percentage of apoptotic spleen and thymus cells, containing a sub-G0 amount of DNA, was determined by flow cytometry after staining the DNA of permeabilized cells with a staining buffer containing 10 μg/ml propidium iodide, 0.01% RNAse,

0.1% Na-citrate and 0.1% Triton X-100 (SIGMA, St. Louis, MO). In another series of experiments, apoptotic cells were detected according to their staining with FITC labeled Annexin V (Roche Biosciences, Indianapolis, IN) and 1 μg/ml propidium iodide. Necrotic or late apoptotic cells were differentiated from early apoptotic cells based on their double staining with FITC-Annexin V and propidium iodide. Constant phagocytosis of the apoptotic cells in the lymphoid organs may decrease the number of detectable apoptotic cells. In some of the experiments, the percentage of apoptotic cells was increased by incubating single cell suspensions at 37 °C in complete tissue culture medium – the phagocytosis of apoptotic cells is less effective under these conditions [1]. This treatment indeed increased the number of apoptotic cells, while the tendencies observed analysing fresh cell suspensions did not change.

For the RNAse protection assay, spleens and thymi were frozen on dry ice, then total cellular RNA was prepared with the guanidium isothyocianate method according to standard protocols and the integrity of the RNA samples was checked by running 5 μg RNA on formaldehide gels. Ribonuclease Protection Assay (RPA) was carried out with the ML-11 multiple probe set using 10 μg of total cellular RNA essentially as described earlier [13]. This set detects transcripts of 10 different murine cytokine genes (lymphotoxin α, TNF-α, IL-4, IL-5, IL-1α, IFN-γ, IL-2, IL-6, IL-1β and IL-3) and the housekeeping L-32 gene. In each gel 1/10 part of the total amount of probe and a pooled spleen sample from LPS treated mice have always been included as molecular weight marker and positive control, respectively. RPA gels were visualized and quantified by using a phosphorimager (Molecular Dynamics, Amersham Biosciences, Little Chalfont, UK).

Tumor growth experiments

Cultured B16 metastatic melanoma cells, obtained originally from a lung metastasis nodule, were injected i.m. into the left hind thigh of female B6 mice (10^5/mouse). After 4 weeks, the animals were over-anesthetized by ethyl-ether, their body weight was measured, tumor diameters were determined by a caliper, the lungs were removed and put in Petri dishes with PBS, and then macroscopic lung metastatic nodules were counted.

Statistical analysis

The differences in the number of splenocytes and thymocytes, the percentage of apoptotic cells, the percent NK lysis, the percentage of NK cells, the tumor diameters and the average lung metastasis numbers were analyzed by two-tailed t-tests in Microsoft Excel. The differences in the ratios of metastasis-bearing mice in the different experimental groups were analyzed by Fisher's Exact test by the GraphPad Instat statistical software.

RESULTS

Our standard stress protocol, 2 × 12 hr immobilization overnight, did not cause significant, reproducible changes in the spleen and thymus cell number, cell composition or apoptosis in stressed B6 mice. A representative example of these experiments is shown in Fig. 1. Analogous experiments performed in DBA/2 mice gave similar results. In accordance with these data, no differences were detected in the percentage of the splenic NK cells by flow cytofluorimetry (data not shown).

The NK activity of spleen cells of stressed and control B6 mice did not differ either. In the demonstrative example shown in Fig. 2, there was no significant difference at a high effector/target ratio, while an unexpected difference was visible at low effector/target ratio, where the specific lysis values were higher in the case of the stressed animals than in the non-stressed controls.

We encountered a similar lack of effect of restraint studying the level of cytokine mRNA-s in the spleen and thymus of stressed and control mice. The same lack of effect was evident on the TNF-α and IL-6 production of LPS stimulated spleen cells.

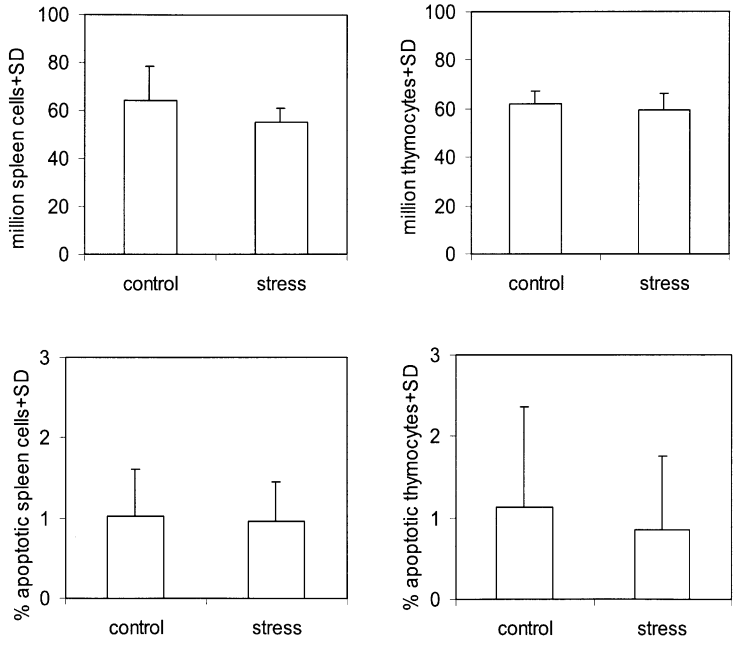

Fig. 1. Lack of effect of immobilization stress on spleen and thymus cell content and apoptosis. Upper left panel: spleen cell number of immobilized and control mice. Upper right panel: thymus cell number of immobilized and control mice. Lower left panel: percentage of apoptotic splenocytes (cells with a sub-G0 DNA content, as measured by flow cytofluorometric analysis of permeabilized, propidium-iodide stained cells) in immobilized and control mice. Lower right panel: percentage of apoptotic thymocytes in immobilized and control mice. No significant differences were observed (n = 4, controls, n = 5, immobilized)

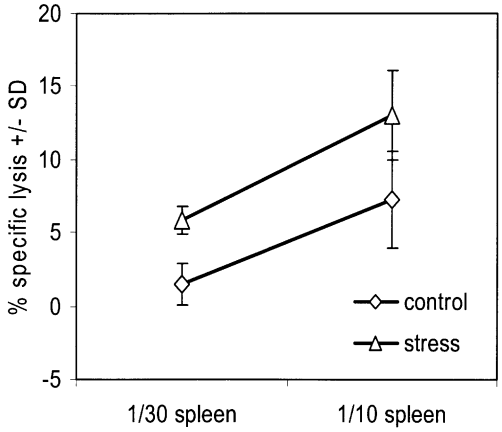

Fig. 2. NK activity of spleen cells of immobilized and control mice. MitoTracker labelled HeLa NK target cells were incubated with different numbers of spleen cells (1/10 or 1/30 of a spleen) of stressed (n = 4) and control (n = 3) mice, then the samples were stained with propidium iodide and live and dead target cells were enumerated by flow cytofluorometry

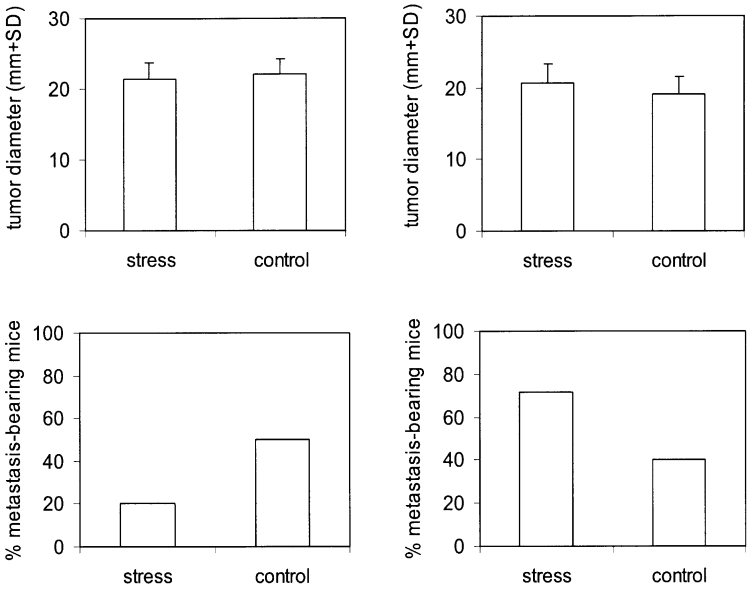

Fig. 3. Growth of primary tumors and lung metastasis formation in immobilized and control mice. B6 mice were injected i.m. in the hind leg with B16 melanoma cells, then the growth of the primary tumors and lung metastases was analysed. The mice were exposed to immobilization stress immediately before the tumor injection (upper left and lower left panel) or one week before (upper right and lower right panel). None of the differences were statistically significant

In addition, in vivo treatment with the opioid receptor antagonist naloxone [4] did not change the level of cytokine mRNAs in the spleen of stressed mice (data not shown).

Our pilot experiments performed with other stress protocols (2, 12 or 24 hr immobilization, spatial disorientation, social conflict, weaning) gave similar results. Most experiments were repeated in both B6 and DBA/2 mice. The two mouse strains were chosen for the experiments because they are known for their obviously different behavior and immunological characteristics (for references see the web site of Jackson Laboratories, *www.jax.org*). Taken together, in our hands, both mouse strains seemed to be refractory to the stress-induced immunomodulation.

In accordance with the lack of significant immunological changes, no reproducible effect was found in our *in vivo* tumor growth experiments either. Since psychological stress is supposed to possess immediate, as well as long-term immunological effects, two stress protocols were used. Some of the mice were injected with tumor cells immediately after the second immobilization, while others one week later. The restraint did not influence significantly the body weight of the tumor bearing mice, the growth of primary tumors, or the percentage and average size of lung metastases in either case (Fig. 3).

DISCUSSION

Taken together, we observed that our experimental animals, kept in an average general purpose animal house, were practically resistant to stress induced immunomodulation, furthermore, their resistance to tumor growth and metastasis formation remained essentially unchanged. We suppose that a high number of similar studies, demonstrating a lack of correlation between a supposedly drastic psychological stressor and the immune response, were performed and remained unpublished because of their nonconformity with the widespread preconceptions. In fact, publications reporting negative results in stress immunology models are very rare [10, 11]. According to our opinion, publication of such studies would be necessary for demonstrating the high variability and low reproducibility of the phenomenon of stress-induced immunomodulation in rodent models.

A major problem what we have encountered screening the literature of mouse ethology is that the *Materials and methods* sections of most stress-immunological papers are not adequately detailed in the description of the animal house and the experimental conditions. It is an important point, since now we believe that stress-induced immunomodulation is demonstrable only in mice grown up isolated from any background stressors, or rather stimuli.

Some of the potential background stressors are quite well known. The detrimental effect of high noise level has been known for a very long time [18]. The presence of predator olfactory clues, for example, rat odor [5] is also known to induce anxiety in mice. It is less widely known that pheromones released by stressed con-species can also influence the stress status of the animals [6]. In the case of some other stressors the situation is much more complex. Isolation from the colony is obviously a part of

most stress protocols. Isolation of mice from their cage-mates is regarded as a stressor by most investigators, but behavioral studies measuring the anxiety parameters of mice revealed that isolation might have opposing effects on male and female mice. According to these data, isolation generates anxiety in female mice, but, being the equivalent of gaining and defending a breeding territory, it is a securising factor for the rather territorial males [21]. It is very probable that surprisingly weak background stimuli may function as stressors, being sufficient for diminishing the effect in psychological stress. Large rooms, white walls, changes in the temperature or the diurnal light level, abrupt noises may adversely influence the stress status of experimental mice, as measured by assays of behavioral conditiong, stress-induced anxiety or hystochemical demonstration of protein phosphorylation in the hippocampus [R. Maldonado, F. Papaleo, personal communications]. Small, dark animal rooms, tightly controlled temperature, stable 12-hour light-dark cycle, absence of sound – not only audible, but ultrasound – stimuli (mice are sensitive to ultrasound frequencies up to 80 000 Hz [15]), absence of other animal species, especially natural predators of mice, absence of contact with non-littermate mice, absence of stress pheromones of mice exposed to stressors (sufficient distance from the animal experimentation rooms, operating rooms, or animal rooms when other mice are exposed to stressors), regular contact with the person who will perform the experiment prior to the manipulation, but no contact with unfamiliar persons, are all necessary for eliminating the background stress. These requirements might easily be overlooked by investigators following protocols in published papers of the field of stress immunology.

At this point, the question arises: whether this lack of stimuli can be regarded as natural state, modeling the everyday life of a mouse – or a human being? Enriched environment, which is on the way of becoming a standard, may make mice more resistant to stressor-induced anxiety – in other words, a more natural level of background stimuli makes animals healthier from a psychological point of view, and it may elevate their psychological stress "threshold" to a normal level [24, 29]. This should be taken into account when evaluating the results of stress immunology experiments.

In conclusion, it seems highly probable that stress-induced immunomodulation is a phenomenon specific to animals grown up in an artificially isolated, stimulus-free, non-enriched environment, very unlike the natural environment of wild animals, so the relevance of the phenomenon from an evolutionary point of view, as well as its relevance to human clinical situations, should be reconsidered.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Hungarian Ministry of Welfare (ETT 39/2000). The ML11 primer set was a kind gift of M. V. Hobbs.

REFERENCES

1. Bercovici, N., Cambouris, C., Vizler, C., Heurtier, A., Liblau, R. (2000) Systemic administration of agonist peptide blocks the progression of spontaneous CD8-mediated autoimmune diabetes in transgenic mice without bystander damage. *J. Immunol. 165,* 202–210.
2. Bonneau, R. H., Sheridan, J. F., Feng, N. G., Glaser, R. (1991) Stress-induced effects on cell-mediated innate and adaptive memory components of the murine immune response to herpes simplex virus infection. *Brain Behav. Immun. 5*, 274–295.
3. Bonneau, R. H., Sheridan, J. F., Feng, N. G., Glaser, R. (1991) Stress-induced suppression of herpes simplex virus (HSV)-specific cytotoxic T lymphocyte and natural killer cell activity and enhancement of acute pathogenesis following local HSV infection. *Brain Behav. Immun. 5,* 170–192.
4. Borsodi, A. (1991) What is the basis of opioid receptor types and the interaction between them? In: Bashbaum, A. I., Besson, J. M. (eds), *Towards a new pharmacotherapy of pain.* John Wiley and Sons, Ltd., 241–255.
5. Calvo-Torrent, A., Brain, P. F., Martinez, M. (1999) Effect of predatory stress on sucrose intake and behavior on the plus-maze in male mice. *Physiol. Behav. 67*, 189–196.
6. Cocke, R., Moynihan, J. A., Cohen, N., Grota, L. J., Ader, R. (1993) Exposure to conspecific alarm chemosignals alters immune responses in BALB/c mice. *Brain Behav. Immun. 7,* 36–46.
7. Dhabhar, F. S., McEwen, B. S. (1996) Stress-induced enhancement of antigen-specific cell-mediated immunity. *J. Immunol. 156,* 2608–2615.
8. Dhabhar, F. S., Miller, A. H., McEwen, B. S., Spencer, R. L. (1995) Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms. *J. Immunol. 154,* 5511–5527.
9. Dhabhar, F. S., Satoskar, A. R., Bluethmann, H., David, J. R., McEwen, B. S. (2000) Stress-induced enhancement of skin immune function: A role for gamma interferon. *Proc. Natl. Acad. Sci. USA 97,* 2846–2851.
10. Flint, M. S., Tinkle, S. S. (2001) C57BL/6 mice are resistant to acute restraint modulation of cutaneous hypersensitivity. *Toxicol. Sci. 62,* 250–256.
11. Flores, C. M., Hernandez, M. C., Hargreaves, K. M., Bayer, B. M. (1990) Restraint stress-induced elevations in plasma corticosterone and beta-endorphin are not accompanied by alterations in immune function. *J. Neuroimmunol. 28,* 219–225.
12. Fukui, Y., Sudo, N., Yu, X. N., Nukina, H., Sogawa, H., Kubo, C. (1997) The restraint stress-induced reduction in lymphocyte cell number in lymphoid organs correlates with the suppression of in vivo antibody production. *J. Neuroimmunol. 79*, 211–217.
13. Hobbs, M. V., Weigle, W. O., Noonan, D. J., Torbett, B. E., McEvilly, R. J., Koch, R. J., Cardenas, G. J., Ernst, D. N. (1993) Patterns of cytokine gene expression by CD4+ T cells from young and old mice. *J. Immunol. 150,* 3602–3614.
14. Ishihara, Y., Matsunaga, K., Iijima, H., Fujii, T., Oguchi, Y., Kagawa, J. (1999) Time-dependent effects of stressor application on metastasis of tumor cells in the lung and its regulation by an immunomodulator in mice. *Psychoneuroendocrinology 24,* 713–726.
15. Koay, G., Heffner, R. S., Heffner, H. E. (2002) Behavioral audiograms of homozygous medJ mutant mice with sodium channel deficiency and unaffected controls. *Hearing Res. 171,* 111–118.
16. Kurihara, H., Koda, H., Asami, S., Kiso, Y., Tanaka, T. (2002) Contribution of the antioxidative property of astaxanthin to its protective effect on the promotion of cancer metastasis in mice treated with restraint stress. *Life Sci. 70,* 2509–2520.
17. Li, T., Harada, M., Tamada, K., Abe, K., Nomoto, K. (1997) Repeated restraint stress impairs the antitumor T cell response through its suppressive effect on Th1-type CD4+ T cells. *Anticancer Res. 17,* 4259–4268.
18. Monjan, A. A., Collector, M. I. (1977) Stress-induced modulation of the immune response *Science 196,* 307–308.
19. Naftzger, C., Takechi, Y., Kohda, H., Hara, I., Vijayasaradhi, S., Houghton, A. N. (1996) Immune response to a differentiation antigen induced by altered antigen: A study of tumor rejection and autoimmunity. *Proc. Natl. Acad. Sci. USA 93,* 14809–14814.

20. Oya, H., Kawamura, T., Shimizu, T., Bannai, M., Kawamura, H., Minagawa, M., Watanabe, H., Hatakeyama, K., Abo, T. (2000) The differential effect of stress on natural killer T (NKT) and NK cell function. *Clin. Exp. Immunol. 121,* 384–390.
21. Palanza, P., Gioiosa, L., Parmigiani, S. (2001) Social stress in mice: gender differences and effects of estrous cycle and social dominance. *Physiol. Behav. 73,* 411–420.
22. Pruett, S. B., Fan, R. (2001) Quantitative modeling of suppression of IgG1, IgG2a, IL-2, and IL-4 responses to antigen in mice treated with exogenous corticosterone or restraint stress. *J. Toxicol. Environ. Health A. 62,* 175–189.
23. Roy, S., Loh, H. H. (1996) Effects of opioids on the immune system. *Neurochem. Res. 21,* 1375–1386.
24. Roy, V., Belzung, C., Delarue, C., Chapillon, P. (2001) Environmental enrichment in BALB/c mice: effects in classical tests of anxiety and exposure to a predatory odor. *Physiol. Behav. 74,* 313–320.
25. Salzet, M., Vieau, D., Day, R. (2000) Crosstalk between nervous and immune systems through the animal kingdom: focus on opioids. *Trends Neurosci. 23,* 550–555.
26. Vizler, C., Nagy, T., Kusz, E., Glavinas, H., Duda, E. (2002) Flow cytometric cytotoxicity assay for measuring mammalian and avian NK cell activity. *Cytometry 47,* 158–162.
27. Wang, J., Charboneau, R., Barke, R. A., Loh, H. H., Roy, S. (2002) μ-opioid receptor mediates chronic restraint stress-induced lymphocyte apoptosis. *J. Immunol*. 169, 3630–3636.
28. Wiltrout, R. H., Herberman, R. B., Zhang, S. R., Chirigos, M. A., Ortaldo, J. R., Green, K. M. Jr, Talmadge, J. E. (1985) Role of organ-associated NK cells in decreased formation of experimental metastases in lung and liver. *J. Immunol. 134,* 4267–4275.
29. Würbel, H. (2001) Ideal homes? Housing effects on rodent brain and behaviour. *TRENDS in Neurosci. 24,* 207–211.
30. Yang, E. V., Glaser, R. (2002) Stress-induced immunomodulation and the implications for health. *Int. Immunopharmacol. 2,* 315–324.
31. Yin, D., Tuthill, D., Mufson, R. A., Shi, Y. (2000) Chronic restraint stress promotes lymphocyte apoptosis by modulating CD95 expression. *J. Exp. Med. 191,* 1423–1428.