Effect of Differential Housing in Mice on Natural Killer Cell Activity, Tumor Growth, and Plasma Corticosterone (43366)

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Abstract. **Various forms of stress have been shown to alter natural killer (NK) cell activity and tumorigenesis; however, few studies have measured these two variables simultaneously. Isolation of mice was utilized as a model of stress by which to study NK cell activity and pulmonary metastatic response following a tumor challenge. Male C3H mice were group or individually housed for 3 weeks, after which CIRAS 3 fibrosarcoma tumor cells or the tumor vehicle was injected intravenously (tail vein), NK cell activity, pulmonary metastasis, and plasma corticosterone were measured 1, 7, and 21 days following tumor cell inoculation. Individually housed mice, irrespective of tumor or vehicle condition, had a higher NK response on Day 1 relative to group-housed animals ($P < 0.001$). By Day 21, tumor condition, rather than housing, was the major significant factor affecting NK activity ($P < 0.001$). Nevertheless, individually housed, tumor-injected mice still had higher NK activity compared with the other treatment groups on Day 21. No effect of housing condition was present for the incidence of pulmonary metastases or frequency of metastases in affected animals. Plasma corticosterone levels generally increased over the study period, with no housing or injection effects at Days 1 and 7. Individually housed, vehicle-injected mice had higher corticosterone levels at Day 21 ($P < 0.01$). These data suggest that in response to housing condition, NK cell activity differs in tumor-bearing mice and vehicle controls. Furthermore, CIRAS 3 pulmonary tumor formation is not affected by differences in NK activity consequent to housing condition. Plasma corticosterone does not appear to be a major *in vivo* regulator of NK activity in this experimental tumor system. Finally, the interpretation of housing effects on NK activity and plasma corticosterone levels depends on the temporal window in which sampling occurs.** [P.S.E.B.M. 1992, Vol 199]

Since ancient times people have believed that stress and the ability to cope with stress may influence disease processes (1). More recent attention has focused on the relationship between stress and cancer. For example, stress and coping skills have been correlated with prognosis in breast cancer (2, 3) and malignant melanoma (4). Experimental tumor growth in mice (5) and in rats (6) exposed to stressful stimuli is influenced by the animal's degree of control over the stress. Stress and the behavioral response to stress may also play a role in long-term cancer risk (7).

Although the mechanisms underlying the putative relationship between stress and cancer are poorly defined, both hormonal and immunological factors have been suggested (8, 9). A variety of physical and psychological stressors can affect both nonspecific (innate) and specific immunity, the direction and magnitude depending upon the chronicity of the stress and the degree of controllability (10–13). Endocrine changes accompanying stress have been well documented, especially for catecholamines and glucocorticoids (14, 15). Interactions between the immune system and the pituitary-adrenal axis (16) lend conceptual support for physiologically relevant mechanisms underlying the relationship between stress and cancer.

Animal models used to investigate stress and cancer suggest a role for housing condition modifying tumorigenesis (17). However, the direction of the effects is by no means consistent (18, 19). Variations in tumor outcome, as a function of the housing condition of the

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animal, depend on many factors, including specific characteristics of the tumor, duration of exposure to housing stress (isolation or crowding), and coping skills of the animals. Housing condition is also known to affect immune function. Individually housed mice had higher specific immune responses (6) and higher macrophage activity (20) than did group-housed mice. Mouse natural killer cell activity was reported to be unaffected by long-term differential housing of the animals, although an acute change in housing modulated the response (21). Few studies have investigated experimental tumorigenesis and immune parameters under conditions of differential housing stress.

The aim of the present experiments was to determine the effects of group and individual housing on natural killer (NK) cell activity during experimental tumorigenesis. NK cells are a group of non-T, non-B lymphocytes that have the unique ability to lyse a variety of tumor targets without prior sensitization and that may play an important role in the control of tumor metastasis (22). NK cells probably represent a first line of defense against neoplasia (23). It is reasonable to assume, then, if differences in tumorigenesis between group and individually housed animals have an immunological component, NK cells may be involved. In addition, because differential housing elicit different stress hormone profiles (24) which in turn could influence NK activity, plasma corticosterone levels were also monitored.

Methods

Animals. One hundred and forty-two male C3H/He mice (21.2–29.3 g), obtained from Charles River (St. Constant, Quebec), were maintained on a reversed 12:12-hr light:dark cycle at an ambient temperature of 23 ± 1°C in opaque polypropylene cages (24 × 16 × 13 cm). Mice were allowed *ad libitum* access to food (Ralston Purina Rodent Chow) and tap water throughout the study. The mice were weighed weekly and examined periodically for signs of disease or injury. All mice were acclimated to the laboratory conditions for 2 weeks prior to the experimental treatment. During the acclimation period, mice were housed in groups of four or five per cage.

Treatment Conditions. Mice were randomly assigned to group housing ($n = 4$ per cage) or to individual housing conditions at the end of the acclimation period; a total of 72 mice were group housed and 70 were individually housed. Within each housing condition, mice were further randomized into tumor injection (group: $n = 38$; individual: $n = 36$) or vehicle injection (group: $n = 34$; individual: $n = 34$). Finally, within each housing and injection condition, mice were randomly assigned to one of three sampling times (1, 7, or 21 days after injection: $n = 11$ per condition in vehicle-injected mice and $n = 12$ in tumor-injected mice; one extra mouse was assigned to the tumor condition because of potentially higher mortality in those animals). After 3 weeks, mice were injected intravenously with tumor cells or vehicle. Mice were sacrificed 1, 7, or 21 days after the injections by exposure to carbon dioxide. All animals appeared to be healthy and in no apparent distress throughout the study.

Tumor. The CIRAS 3 tumor used in this study is an H-*ras*-transformed 10T½ murine fibroblast line originally derived from C3H 10T½ cells transfected with the pAL8A plasmid; the CIRAS 3 cells were a gift from Dr. A. Greenberg (Manitoba Institute of Cell Biology). This tumor line is known to be sensitive to lysis by NK cells. Aliquots of tumor cells (1×10^6) were kept frozen at −80°C in dimethyl sulfoxide until use. The growing tumor cell line was maintained in tissue culture medium (Dulbecco's modified Eagle's medium-F12 medium) supplemented with 10% fetal calf serum and 50 units/ml of penicillin. Cell cultures were grown at 37°C in a humidified 5% CO_2 atmosphere.

Experimental Metastasis Assay. Metastatic potential was assayed by the experimental metastasis assay (25) in which 3×10^5 tumor cells were injected into the tail vein of mice. Tumor cells were harvested from subconfluent cultures in the exponential growth phase by overlaying the cells with a thin layer of 0.5% trypsin and 0.2% EDTA for 10 min to ensure detachment. The petri dish was tapped sharply to dislodge the cells, and tissue culture medium was immediately added. The cells were then washed and resuspended in Hanks' balanced salt solution at a concentration of 1.5×10^6 cells/ml. Tumor cell viability was about 95% based upon the ability of tumor cells to exclude trypan blue.

Mice were injected with 200 μl of CIRAS 3 (3×10^5 cells) or an equal volume of Hanks' balanced salt solution vehicle. Injections were performed by gently warming the tail under a heating lamp in order to dilate the tail vein. All injections took place between 0800 and 1100 hr. All injections were completed without difficulty with the full volume of tumor cell suspension being administered to the appropriate animals. Mice were sacrificed 1, 7, or 21 days after injection for determination of tumor metastasis. Intratracheal perfusions with Bouin's solution were done in order to facilitate visualization of lung tumor nodules. The number of lung surface tumor foci was determined under a dissecting microscope.

Splenic Effector Cell Suspensions. Spleens excised from mice were made into single-cell suspensions by pressing through fine gauze mesh with a syringe. The preparation was suspended in RPMI 1640 medium supplemented with 2 m*M* L-glutamine, 10% heat-inactivated fetal bovine serum, 50 units/ml of penicillin-streptomycin mixture, 2.5×10^{-5} *M* 2-mercaptoethanol, and 10 m*M* HEPES buffer. Erythrocytes were lysed with distilled water, and the remaining cell suspension

was washed with 2 × phosphate-buffered saline and resuspended in RPMI 1640 at a concentration of 1.5×10^7 cells/ml for the microcytotoxicity assay.

Target Cells. The YAC-1 cell line, a Moloney leukemia virus-induced lymphoma of the A/Sn strain of mouse, was maintained in a suspension in RPMI 1640 medium supplemented with penicillin, streptomycin, glutamine, and 10% heat-inactivated fetal bovine serum. YAC-1 were used as highly sensitive targets for NK-mediated cytolysis *in vitro.*

Cytotoxicity Assay. A standard microcytotoxicity assay was used as described previously (13). Briefly, target cells were labeled with [^{51}Cr] sodium chromate, washed, and distributed into V-bottomed, 96-well microtiter plates at a concentration of 10^4 cells/100 μl. Effector spleen cells at various effector to target cell ratios ranging from 150:1 to 18:1 were added in 100 μl volumes in triplicate to the plated labeled target cells. For the spontaneous ^{51}Cr release control, 100 μl of medium were added to the labeled target cells. For the total ^{51}Cr release control, 100 μl of Triton X-100 (detergent) were added to the labeled target cells. Microtiter plates were incubated at 37°C in a 5% CO_2 environment for 4 to 6 hr. After incubation, the plates were centrifuged at 400*g* for 10 min, and 100 μl of the supernatant were removed from each well and counted on a Beckman 5500 gamma counter. Data were expressed as percentage of specific ^{51}Cr release:

$$\frac{\text{cpm test sample} - \text{cpm spontaneous release}}{\text{cpm total release} - \text{cpm spontaneous release}} \times 100\%$$

Corticosterone Assay. Cardiac blood was collected immediately upon sacrifice; care was taken to ensure that less than 3 min elapsed between handling the animal for sacrifice and removal of cardiac blood. Blood was then centrifuged at 2500*g* for 15 min at 4°C and the plasma was removed and stored until use at −80°C. Plasma corticosterone concentrations were determined using a commercially available [^{125}I]corticosterone radioimmunoassay (ICN, Immunocorp, Montreal, Quebec). The kit did not require an extraction step for plasma.

Statistical Analysis. Data were analyzed with SYSTAT: The System for Statistics for the PC (26). NK cell activity, plasma corticosterone, and body weight data were each analyzed by three-way analysis of variance (housing, injection, and sampling time), factorial design. Within each sampling time, NK data were also analyzed by analysis of variance with repeated measures (to determine effects at the four effector to target cell ratios). Differences between means of treatment groups were tested post hoc with Tukey's test. Tumor data were analyzed nonparametrically (Kruskal-Wallis analysis of variance). Statistical significance was set at a *P*-value of 0.05.

Results

Figure 1 shows the baseline NK activity at an effector to target cell ratio of 150:1 by the various treatment and time conditions. Overall, there was a highly significant interaction effect observed between injection condition and sampling point ($P < 0.0001$) and a marginally significant interaction between housing and injection condition ($P < 0.047$). Generally, NK cytotoxicity increased from the first to the final sampling point and most markedly in the tumor-injected mice. In mice sacrificed 1 day after injection of tumor or vehicle, housing condition affected the magnitude of the NK response ($P < 0.001$). Individually housed mice had higher NK cytolytic activity than did group-housed mice, an effect largely due to the elevated response in the tumor-injected animals. At 7 days after injection, there was no overall main effect of housing or injection condition. All treatment groups, except the individually housed, tumor-injected group, had higher NK cytolytic responses at 7 days compared with 1 day; this increase in activity over time was statistically significant for group-housed, vehicle-injected mice (Tukey's test, $P < 0.003$) and marginally nonsignificant for individually housed, vehicle-injected mice (Tukey's test, $P > 0.08$). In mice sacrificed at 21 days, injection treatment ($P < 0.001$), but not housing, was significant. Tumor-injected mice had higher NK cell activity, an effect due to the marked increase observed in individually housed animals. Table I lists the percentage of lysis for all ratios at Day 1 and Day 7 for the four treatment groups. Figure 2 shows a representative dose-response curve for splenic NK cell activity (four effector to target cell ratios) in mice sacrificed 21 days after injection of tumor or vehicle.

The number of lung tumors per mouse and the frequency of affected mice 21 days after injection of CIRAS 3 are shown in Table II. There were no significant differences in either the mean number of tumors or in the number of affected animals by housing condition.

Plasma corticosterone concentrations are shown in Figure 3. There was a significant interaction between housing and sampling time on plasma corticosterone ($P < 0.016$), with corticosterone levels tending to be higher at 21 days than at 1 day or 7 days. At 1 day after injection of tumor cells or vehicle, group-housed mice had higher plasma corticosterone levels (group tumor: 19.7 ± 10.3 ng/ml; group vehicle: 12.0 ± 4.0 ng/ml) than did individually housed mice (individual tumor: 7.2 ± 2.4 ng/ml; individual vehicle: 7.7 ± 2.7 ng/ml); however, these differences were not statistically significant. There were also no significant differences in the plasma corticosterone concentrations across groups at

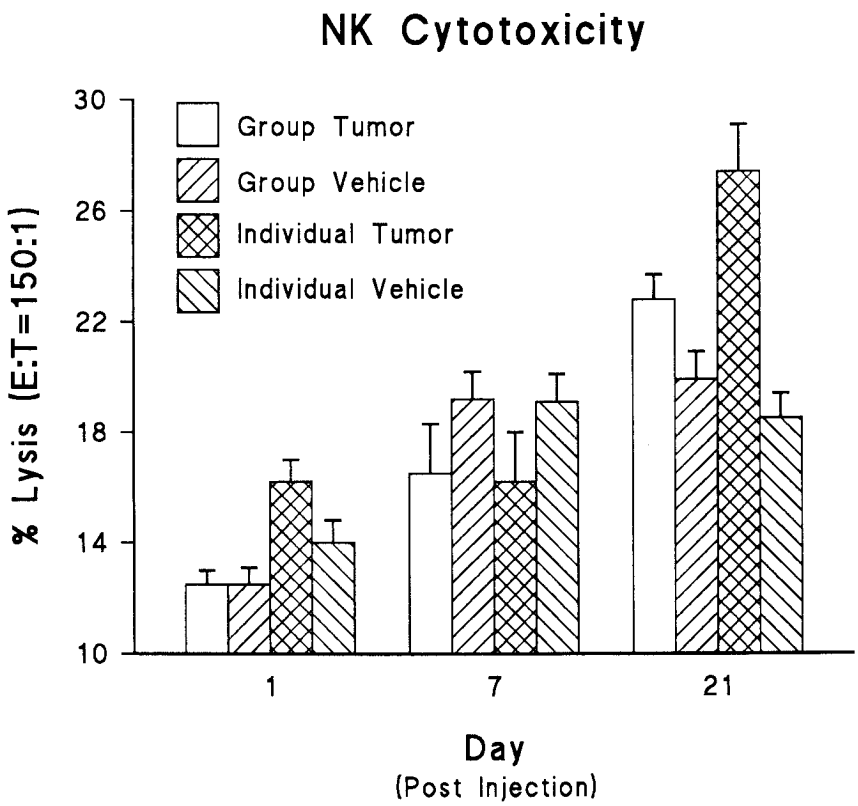

Figure 1. Percentage of NK cytotoxicity (effector to target cell ratio = 150:1) in mice following intravenous (tail vein) injection of 3×10^5 CIRAS 3 tumor cells or vehicle displayed by housing and injection condition at various times after injection.

Table I. Percentage of NK Cytotoxicity in Mice[a]

Group	Effector to target ratio			
	18:1	37:1	75:1	150:1
Day 1				
GT	5.0 ± 0.2	6.6 ± 0.3	9.3 ± 0.4	12.5 ± 0.5
IT	7.8 ± 0.2	9.3 ± 0.2	12.2 ± 0.3	16.2 ± 0.8
GV	4.7 ± 0.4	6.8 ± 0.3	9.5 ± 0.4	12.5 ± 0.6
IV	6.4 ± 0.7	8.2 ± 0.6	11.3 ± 0.7	14.0 ± 0.8
Day 7				
GT	5.4 ± 0.7	7.5 ± 1.0	11.7 ± 1.4	16.5 ± 1.8
IT	5.2 ± 0.7	7.3 ± 0.9	11.5 ± 1.4	16.2 ± 1.8
GV	5.3 ± 0.5	8.5 ± 0.6	13.4 ± 0.8	19.2 ± 1.0
IV	5.1 ± 0.5	8.3 ± 0.6	13.1 ± 0.8	19.1 ± 1.0

[a] The percentage of NK cytotoxicity in mice was determined after intravenous (tail vein) injection of 3×10^5 CIRAS 3 tumor cells or vehicle displayed by housing and injection condition at Day 1 and Day 7 after injection. Values are mean ± SE. GT: group housed, tumor bearing; IT: individually housed, tumor bearing; GV: group housed, vehicle treated; IV: individually housed, vehicle treated.

the 7-day sampling point. In contrast, at 21 days after injection, housing conditions strongly influenced corticosterone levels ($P < 0.01$). Individually housed mice had higher plasma corticosterone levels (50.6 ± 7.3 ng/ml collapsed across injection treatments) than did group-housed mice (26.4 ± 4.9 ng/ml collapsed across injection treatments). This effect was primarily due to the marked increase in corticosterone levels in vehicle-injected, individually housed animals. In addition, plasma corticosterone concentrations in the individually housed, vehicle-injected mice were higher at day 21 sampling than at the earlier time points (Tukey's test; vs 1 day: $P < 0.001$; vs 7 days: $P < 0.01$).

Changes in body weight (final-initial) displayed a significant effect of housing ($P < 0.0001$) and sampling time ($P < 0.0001$). Table III shows the body weight changes in mice as a function of housing and injection. Individually housed mice gained the least weight and group-housed mice gained the most weight over the duration of the study. Initially, group-housed mice weighed slightly less than individually housed mice, but this difference was no longer present at the end of the study.

Discussion

A central dogma in the field of stress biology is that individual housing (isolation) produces major behavioral and physiological, and especially neuroendocrine, changes in rodents relative to the "normative" condition of group housing. Assumptions underlying the isolation stress model in mice and rats have been reviewed by Brain (27) and Brain and Benton (28). Recent reports also suggest that differential housing in rodents influences various components of the immune system (6, 20, 29) and experimental tumor growth (17,

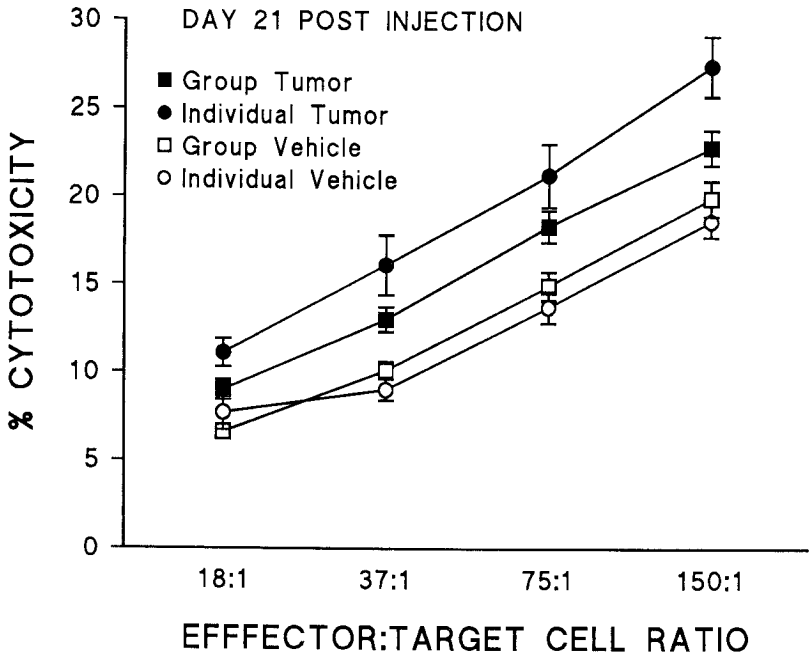

Figure 2. Percentage of NK cytotoxicity at all effector to target cell ratios in mice following intravenous (tail vein) injection of 3×10^5 CIRAS 3 tumor cells or vehicle displayed by housing and injection condition 21 days after injection.

Table II. Number of Lung Tumors and Frequency of Occurrence in Group and Individually Housed Mice

Housing condition	No. of tumors[a]	Frequency
Grouped	38 ± 15	6/11
Individual	42 ± 11	8/11

[a] Lung tumor nodules were counted 21 days after intravenous injection of 3×10^5 CIRAS 3 cells into male C3H/He mice. Values are mean ± SE.

30). Surprisingly, the question of physiological relevance, in the case of housing and immune function studies on the one hand, and immunological mechanisms, in the case of housing and tumor growth studies on the other hand, have not been systematically addressed.

The data from this experiment indicate that although housing condition influences natural killer cell immunity in tumor-bearing mice, the immunological impact is marginal in terms of actual CIRAS 3-induced tumor growth. Individually housed, tumor-injected mice had higher *in vitro* NK lysis of YAC-1 target cells than did both individually housed, vehicle-injected and group-housed, tumor-injected mice. Indeed, the natural killer cell response in the individually housed, tumor-bearing animals increased markedly with the temporal process of tumor growth. Nevertheless, individually housed mice had virtually identical numbers of pulmonary tumor foci 3 weeks after tumor injection compared with those animals that had been group housed. This finding raises several possible explanations about the relationship between natural killer cell activity and tumor growth in differentially housed animals. First, it is possible that the magnitude of the immune changes induced by differential housing is too small to have a clinical impact on tumor-growth. Thus, although individually housed, tumor-bearing mice had an increase in natural killer cell activity from 16.2% (Day 1) to 27.4% (Day 21) (an increase of 69%), clinically meaningful effects may be apparent only at much higher ranges of immunopotentiation. Second, final tumor growth was determined at 21 days when gross metastases could be visualized. Whether other measures of tumor burden (micrometastases and host survival) are differentially responsive to social housing condition and control by natural immune mechanisms is not known. An alternative explanation is the possibility that differential housing stress induced resistance of CIRAS 3 tumors to lysis by natural killer cells. In this regard, Sugawara and associates (31) reported that metabolic stress of tumor cells *in vitro* stimulates the synthesis of stress proteins concomitant with the induction of resistance to lysis by killer cells and tumor necrosis factor. It may also be the case, as suggested by Rowse and coworkers (32), that splenic NK activity does not provide an accurate indiction of NK tumorilytic activity at the site of metastasis.

Our data show that the natural killer cell immune response of tumor-bearing mice, either group or individually housed, differs from non-tumor-bearing mice under the same housing condition. As the duration of the housing treatment continued, natural killer cell activity increased in tumor-bearing animals. By contrast, in vehicle-injected, healthy animals, natural killer

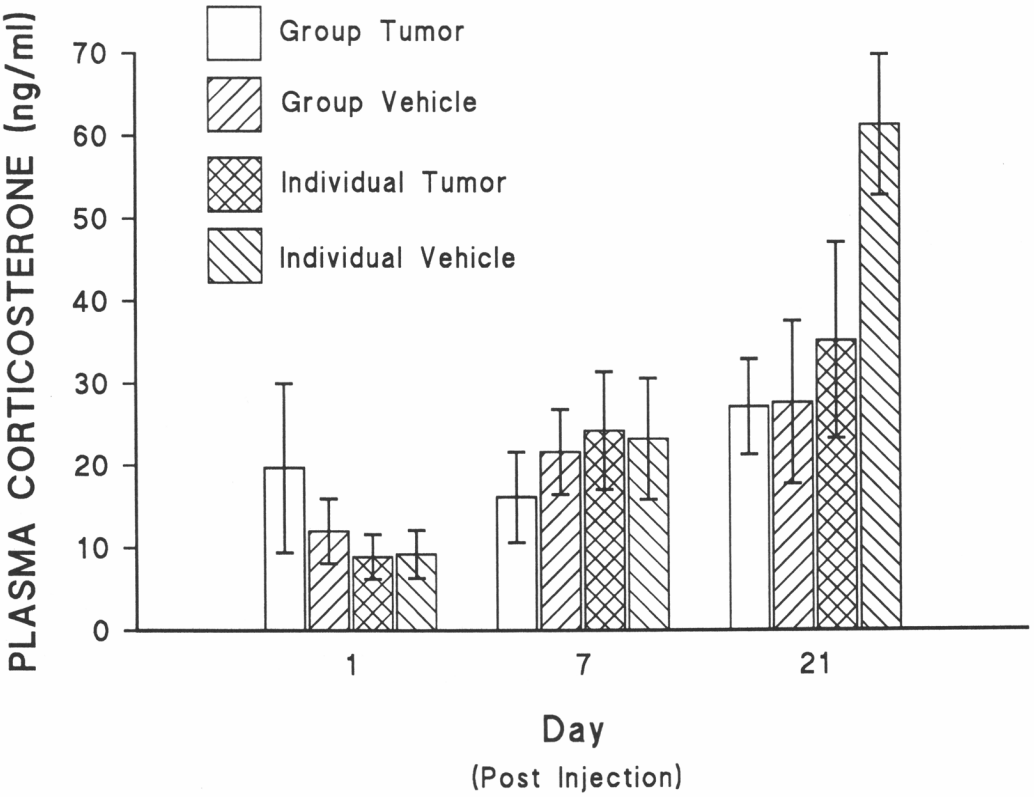

Figure 3. Plasma corticosterone levels in mice following intravenous (tail vein) injection of 3×10^5 CIRAS 3 tumor cells or vehicle displayed by housing and injection condition at various times after injection.

Table III. Change in Body Weights of Group and Individually Housed Mice

Tumor condition	Change in body wt[a] (g)	
	Group housed	Individually housed
Tumor injection	1.7 ± 0.2	1.2 ± 0.2
Vehicle injection	2.2 ± 0.2[b]	1.0 ± 0.2

[a] Final weight in grams minus initial weight in grams.
[b] $P < 0.004$ vs individually housed, vehicle-injected group (Tukey's test).

cell activity initially increased and then plateaued as the duration of the housing treatment continued. Using a model of restraint stress and recovery from restraint, Steplewski and Vogel (11) and Steplewski et al. (33) also found that several immune responses differed in tumor-bearing and non-tumor-bearing rats. Splenic lymphocyte proliferation responses in grouped and individually housed mice, given acute novelty stress, differed in tumor-bearing and non-tumor-bearing animals (18).

In agreement with other investigators (18), housing condition did not differentially affect basal corticosterone levels in tumor-injected mice. Also, both NK activity and plasma corticosterone increased over the 3-week treatment period. These data suggest that the increase in natural killer cell activity in tumor-bearing mice, regardless of housing condition, is not related to adrenocorticoid response. In contrast, individually housed, vehicle-injected mice had elevated basal corticoid levels at the 21-day time point. These findings are at variance with those reported by Jessop and colleagues (12), in which plasma corticosterone levels in rats exposed to 5 weeks of isolation did not differ from group-housed animals. The reasons for the surge in basal corticosterone levels in the individually housed, vehicle-injected mice are not known. Corticosterone levels increase dramatically in rodents in response to acute stress and the marked increase observed in this study is suggestive of such a response. Although care was taken to obtain blood samples for analysis within 3 min of sacrifice, it may be possible that this elevation in corticoid levels is an artifact of slight variations in the timing of blood collection.

The literature on housing stress and immune function suggests an increase in immune parameters in individually housed rodents. Housing stress appears to have just the opposite influence on experimental tumor growth: group-housed animals generally show smaller tumor weights or numbers. In the present study, which addressed differential housing, immune parameters, and tumorigenesis, the impact of differential housing is less evident on innate immunity than is the presence of

tumor. Furthermore, the animals used in these experiments, unlike those in earlier reports (18), were allowed to adjust to the treatment of differential housing for 3 weeks before challenge with tumor. Previously, it has been reported that after a 4-week period, group and individually housed mice did not differ in NK activity (21). Thus, our model was that of chronic stress in which adaptation to the housing conditions may have already occurred by the time tumor was introduced. Whether the interactions among housing condition, innate immunity, and tumorigenesis in a more acute paradigm (i.e., tumor challenge followed by differential housing) differ from the chronic situation described herein remains to be determined. Nevertheless, the early observations made by Jensen (34) regarding the timing of stress exposure in relation to tumor challenge are more than historically interesting.

It is apparent that the response of NK cells to a tumor challenge is a dynamic one. Previous studies have reported a peak in NK activity 3–7 days after injection of tumor cells with a decline in NK activity when measured at later time points (32, 35). Thus, the results observed for any given experiment depend on the window in time through which one chooses to observe the dependent variable. Studies incorporating multiple time points may make interpretations of results more complex, but may offer a better picture of the complete response of the host following a tumor challenge. In summary, the results of this study suggest that tumor-bearing and healthy animals differ in their immunological responses to the background environmental factor of differential housing. The neuroendocrine basis for this difference remains to be determined.

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