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Influence of Change From Grouped to Individual Housing on a T-Cell-Dependent Immune Response in Mice: Antagonism by Diazepam

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SHANKS, N., C. RENTON, S. ZALCMAN AND H. ANISMAN. *Influence of change from grouped to individual housing on a T-cell-dependent immune response in mice: Antagonism by diazepam.* PHARMACOL BIOCHEM BEHAV 47(3) 497-502, 1994.—Transferring CD-1 mice from grouped to individual housing and then maintaining them individually resulted in a decline in the peak IgM plaque-forming cell (PFC) response to sheep red blood cells (SRBCs). However, the immunosuppression was dependent on the amount of time mice were maintained individually. In particular, individual housing for 5-10 days prior to SRBC inoculation and for 4 days following inoculation resulted in a suppression of the splenic PFC response and serum antibody titers. Shorter periods of individual housing (4 days following inoculation) did not provoke the immunosuppression. Likewise, following more protracted individual housing (15-30 days prior to inoculation) the immunosuppression was not evident. Inasmuch as daily treatment with an anxiolytic, diazepam (1.0 mg/kg), antagonized the suppression induced by 5 days of individual housing, it was suggested that the change from group to individual housing and then maintenance of animals individually acted much like a stressor to induce the immunosuppression.

Immunosuppression Individual housing Stress

VARIOUS aspects of the immune response have been shown to be affected by stressors. Among other things, acute stressors may provoke the suppression of natural killer (NK) cell activity (7,27,32) and cell proliferation in response to mitogens (8,17,20), as well as the splenic plaque-forming cell (PFC) response to sheep red blood cells (SRBCs) (18,21,23,33). Likewise, psychosocial events, such as change in housing conditions, may influence immune system activity, as well as the neuroinvasiveness of a virus (5,6,24). It appears, however, that the immunological consequences of psychosocial manipulations, such as individual housing, may be dependent on either the initial social disturbances (i.e., the change from grouped to individual housing conditions) or the length of time in which animals are maintained in the altered social condition. For instance, Hoffman-Goetz, MacNeil, and Arumugam (13) reported that although splenic NK cell activity was increased 1 day after mice were housed individually, this effect was absent after 7 or 21 days of individual housing. The altered NK activity was apparently unrelated to plasma corticosterone concentrations, as the levels of the steroid were elevated in mice housed individually for 21 days, but not in mice housed individually for shorter intervals.

Like NK cytotoxicity, social housing conditions were shown to influence the splenic PFC response. For instance, Vesey (30) observed that five days of individual housing in newly weaned mice resulted in an increase of the antibody response to bovine serum albumin administration. Edwards (10) similarly observed that individual housing enhanced the antibody response to bovine serum; however, transfer of animals from individual to grouped housing and then back again

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to isolation resulted in a diminution of the antibody response. Thus, it was suggested that although individual housing may be associated with elevated immune activity, changes in the housing environment may diminish the immune response. Finally, Rabin, Lyte, and Hamill (25) demonstrated that individual housing was associated with an increased PFC response to SRBC relative to group-housed animals. Since a similar outcome was not evident when the antigen employed was one that interacted directly with B cells, these investigators suggested the altered SRBC response resulting from the social housing conditions was due to alteration of T cell activity (29).

Commensurate with these studies, it has been reported that individual housing enhanced cell proliferation in response to a mitogen in rats (16) and in mice (24), and increased IL-2 production by T helper cells (24). The latter investigators also observed that isolated housing for 10–14 days (commencing as soon as mice were received from the supplier) resulted in increased resistance to infection with *Candida albicans.* Within three weeks, however, the response to the *C. albicans,* as well as the enhanced cell proliferation in response to a mitogen, were no longer apparent. Evidently, the immunologic effects of the social housing manipulation were transient or were related to alterations of social conditions as opposed to stemming from the specific housing condition in which animals had been maintained or a consequence of shipping from the supplier.

Summarizing, there are ample data suggesting that the conditions under which animals are housed may influence immune functioning. However, the variations of immune activity that are evident may be aligned with changes of housing conditions, and disappear as the social conditions stabilize. Alternatively, it is possible that some housing conditions act like a continuous stressor. Thus, the initial impact of the stressor—in this instance, transferring mice from one social condition to another—leads to reduced immune functioning. However, as in the case of other physical and/or psychological stressors, protracted social disruption leads to compensatory changes, including increased immune activity (2). The present investigation was undertaken to assess whether 1) individual housing of mice that had previously been maintained in groups would be associated with alterations of the splenic PFC response and 2) such an effect could be antagonized by pretreatment with an anxiolytic, which is known to prevent some of the well-documented central neurochemical consequences of stressors, including alterations of norepinephrine and mesolimbic dopamine activity (14,15,19).

EXPERIMENT 1

The effects of individual or group housing on immune status have been shown to be dependent on the length of time the particular social conditions are maintained. Accordingly, a preliminary experiment, using CD-1 mice, assessed the splenic PFC response and serum antibody titers to SRBC following a change in social housing coupled with varying amounts of individual housing.

Materials and Methods

Subjects. A total of 107 male CD-1 mice (Charles River Canada Inc.) were obtained at eight weeks of age and allowed four weeks to acclimatize to the laboratory setting. Mice were housed in groups of five in 27 × 21 × 14-cm polypropylene cages and permitted free access to food and water. During individual housing periods mice were housed alone in their home cage. A 12-h light-dark cycle was employed (light 0700–1900), and all experimental manipulations were conducted between 0800 and 1200.

Procedure. Mice were randomly assigned to eight treatment conditions (n = 9–16/group). Mice of one condition were maintained in groups of five throughout the experiment (i.e., during the four-week acclimatization period, the 30 days prior to inoculation and the 4 days after inoculation), while the remaining groups were individually housed for either 0, 5, 10, 15, 20, 25, or 30 days, after which all animals were inoculated with SRBC (10^6 cells). Four days afterward the spleens were taken to assess the peak PFC response to SRBC, while trunk blood was collected for the determination of antibody titers. Throughout the 4-day period between inoculation and the spleens being taken, the latter seven groups were individually housed, while the group-housed animals were kept with their cage mates. SRBCs were prepared by washing citrated sheep's blood (Woodlyn Laboratories Ltd., Guelph, Ontario, Canada) three times in sterile saline. Animals were immunized IP with 10^6 cells in a volume of 0.10 ml.

PFC assay. Determination of the IgM PFC response was made using a slight modification of the method of Cunningham and Szenberg (9). Mice were decapitated and spleens removed and dispersed to a single cell suspension in Hanks balanced salt solution (HBSS) supplemented with *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer (1.0-M solution), gentamycin sulphate (40 mg/ml) and streptomycin G (5000 units/ml; John's Scientific, Toronto). The spleen cells were washed by centrifugation at 400× g for 10 min and resuspended in 2 ml of medium. The cells were then layered on a Ficoll-Hypaque gradient (density = 1.1) and centrifuged at 700× g for 30 min. After centrifugation, the mononuclear cells at the interface were removed, resuspended in 5 ml HBSS and washed three times at 400× g. Twenty microliters of the cell suspension (at 10^7 cells/ml) were combined with 20 μl of SRBCs (~2.5 × 10^8 cells/ml) and 20 μl of guinea pig complement (absorbed with SRBCs at a ratio of 2 : 1). The suspension was introduced by capillary action into microslides (Canlab, Pointe Claire, Quebec) with dimensions of 0.22 × 4 × 100 mm at a volume of 80 μl. The ends of the microslides were sealed with a 50% paraplast embedding medium and 50% vaseline mixture. The slides were incubated at 37°C for approximately one hour. Plaques were counted by microscopic examination of the microslides (in duplicate) at a magnification of 10×. Data were expressed as PFC/10_6 mononuclear cells.

Determination of antibody titer. Mice were decapitated and trunk blood was collected and allowed to clot. Samples were subsequently centrifuged at 400× g for 10 min and the sera collected and spun at 600× g. Serum complement was then inactivated at 56°C for 30 min. Twofold serial dilutions of inactivated serum, saline, and a 1% SRBC solution was then made in glass microwells. The dilution at which aggregation of SRBC was still evident was considered to be the antibody titer and was expressed in $\log_2$ units.

RESULTS

The group means and the individual PFC responses for each subject are shown in Fig. 1. Spleens of two mice were not included in the analysis owing to errors in the assay procedure. A one-way analysis of variance (ANOVA) assessing the effects of the varying durations of individual housing indicated that the PFC response was influenced by the housing manipulation that was applied, $F(7, 99) = 2.21$, $p < .05$. It appeared that a limited duration of individual housing re-

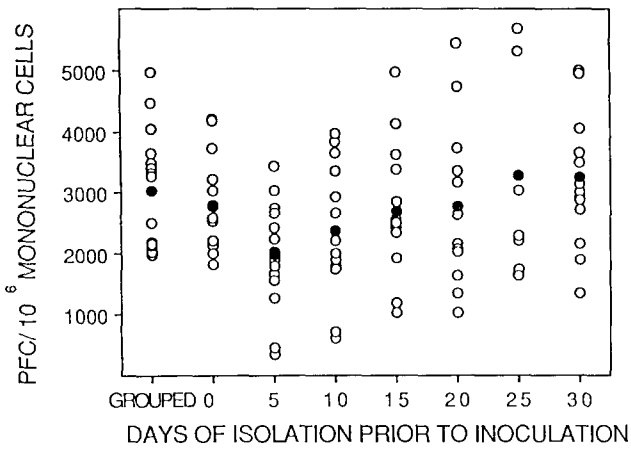

FIG. 1. Individual (○) and the mean (●) splenic plaque-forming cell (PFC) response four days after sheep red blood cell (10^6) treatment as a function of the housing condition. Grouped animals were maintained in groups of four to five throughout. Mice in the individual housing conditions were housed alone for various durations prior to inoculation, as indicated on the abscissa, as well as over the four days between SRBC inoculation and spleens being taken.

sulted in a reduction of the PFC response. However, with continued isolation this reduction was no longer apparent. Specifically, Duncan's multiple range tests ($\alpha = .05$) indicated that 5 days of individual housing prior to inoculation reduced the PFC response relative to group-housed animals or mice that had not been isolated prior to inoculation. As well, 5 days of individual housing prior to inoculation resulted in PFC values lower than those of mice that received either 25 or 30 days of this treatment. Likewise, 10 days of individual housing resulted in a lower PFC response relative to mice that had been maintained in individual housing for 25 or 30 days. The more protracted isolation treatments (15–30 days) did not influence the PFC response in comparison to grouped animals. Paralleling the PFC scores, analysis of the antibody titers (see Table 1) indicated that titers varied as a function of housing condition, $F(7, 101) = 3.93$, $p < .01$. The Duncan's multiple range tests confirmed that both 5 and 10 days of individual housing prior to SRBC inoculation reduced the antibody titers relative to the group-housed animals, as well as to mice that received 0, 25, or 30 days of individual housing prior to SRBC inoculation.

TABLE 1

MEAN ($Log_2 \pm SE$) ANTIBODY TITER VALUES IN MICE EXPOSED TO VARYING AMOUNTS OF INDIVIDUAL HOUSING PRIOR TO INOCULATION

	Mean Antibody Titer
Group-housed	3.37 ± 0.39
0 Days	3.20 ± 0.24
5 Days	1.73 ± 0.28*
10 Days	2.21 ± 0.33*
15 Days	2.57 ± 0.31
20 Days	3.33 ± 0.41
25 Days	3.31 ± 0.37
30 Days	3.13 ± 0.34

*$p < .05$ relative to group-housed mice.

EXPERIMENT 2

It appears that simply a change of housing condition (from group to individual housing) following SRBC inoculation was not sufficient to induce a reduction of the splenic IgM PFC response and serum antibody titers. However, if mice were separated from cage mates for 5–10 days prior to inoculation and maintained in this condition until the time of the peak immune response, then a marked immunosuppression was evident. As indicated earlier, the possibility exists that the immunosuppression may be related to the stress (anxiety or arousal) associated with the change of housing coupled with the isolation procedures. Experiment 2 was conducted to assess whether treatment with diazepam, which is known to antagonize some of the central neurochemical consequences of stressors (14,15,19), would antagonize the effects of the housing manipulation on the splenic IgM PFC response and serum antibody titers.

Materials and Method

A total of 34 male CD-1 mice served as experimental subjects. The subject characteristics and housing procedures were the same as those of experiment 1. Following acclimatization to the laboratory, mice were either housed in groups of four to five or were individually housed. Five days afterwards mice were inoculated with SRBCs (10^6), and four days later spleens and trunk blood were collected for PFC and antibody determinations. Half the mice in each of the housing conditions received IP injections of diazepam (1.0 mg/kg in a volume of 2.5 ml/kg), while the remaining mice received an equivalent volume of vehicle (10% ethanol, 40% propylene glycol, and 50% sterile saline). These treatments were applied on each of the five days (between 0800 and 1000) prior to SRBC inoculation and throughout the four-day period between SRBC administration and subsequent spleen removal.

Results

The mean and individual PFC scores for each of the groups are shown in Fig. 2. The ANOVA of these scores indicated that the PFC values varied as a function of the Housing Condition × Drug Treatment interaction, $F(1, 30) = 12.96$, $p < .01$. Duncan's multiple range tests ($\alpha = .05$) of the means comprising this interaction confirmed that in vehicle-treated mice the PFC response was lower than in mice that had been maintained in groups. In the group-housed animals treatment with diazepam was without effect, whereas isolated animals treated with diazepam exhibited a statistically greater PFC response than the isolated, vehicle-treated mice. Thus, the differences in the PFC response observed between isolated and grouped animals were eliminated by the diazepam treatment.

Commensurate with the data for the antibody-forming cells, ANOVA revealed that the diazepam treatment enhanced the antibody titers, $F(1, 30) = 11.87$, $p < .01$. The Housing Condition × Drug Treatment interaction did not reach statistical significance. However, since a priori predictions had been made concerning this interaction, Newman-Keuls multiple comparisons ($\alpha = .05$) of the simple main effects were conducted. These comparisons confirmed that isolated housing of vehicle-treated mice significantly reduced the serum antibody titers (see Table 2), and this difference was absent in mice that

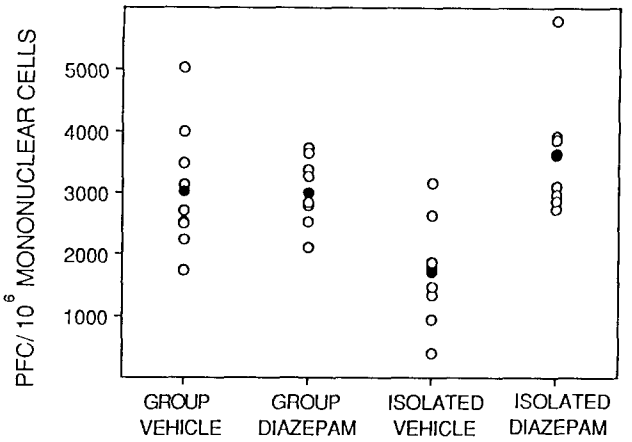

FIG. 2. Individual (○) and the mean (●) splenic plaque-forming cell (PFC) response in mice housed in groups or individually (five days prior to sheep red blood cell inoculation and four days afterward) and treated daily with either diazepam (1.0 mg/kg) or vehicle.

had been treated with diazepam. In group-housed animals the diazepam treatment did not significantly influence antibody titers.

EXPERIMENT 3

The results of the preceding experiment indicated that repeated diazepam treatment effectively eliminated the reduction of the PFC response ordinarily associated with isolated housing. Inasmuch as diazepam did not affect the PFC response of grouped animals, it is unlikely that the effect of the anxiolytic was simply one of enhancing immune activity. In experiment 2 the anxiolytic was administered on each day of individual housing, and it is possible that the drug might have been effective in antagonizing the immunosuppression even if it had been administered acutely only at the time of the peak immune response. That is, the drug may not have been acting to eliminate the anxiety or stress associated with the change of housing (or isolation treatment), but instead may have acted by altering the immune response in mice that ordinarily exhibited suppressed responding. Accordingly, experiment 3 was conducted to ascertain that repeated diazepam treatment was necessary to increase the PFC response and serum antibody titers in mice that had been transferred from grouped to individual housing.

TABLE 2

MEAN (Log_2 + SE) ANTIBODY TITERS OF GROUP- OR INDIVIDUALLY HOUSED MICE TREATED DAILY WITH EITHER DIAZEPAM (1 mg/kg) OR VEHICLE

	Drug Treatment	
Housing Condition	Vehicle	Diazepam
Grouped	2.88 ± 0.24	3.50 ± 0.33
Isolated	2.00 ± 0.46*	3.56 ± 0.18†

*$p < .05$ relative to grouped mice. †$p < .05$ relative to isolated, vehicle-treated mice.

Methods

A total of 49 male CD-1 mice served as subjects in experiment 3. The subject characteristics and the individual housing procedure employed were the same as in experiment 2, except that all animals were individually housed five days prior to SRBC inoculation (10^6 cells). As in the preceding experiments, spleens and blood were collected four days after SRBC treatment. Mice were assigned to five treatment groups and given either daily treatment with diazepam (1.0 mg/kg) or vehicle throughout the nine days of individual housing (five days prior to SRBC and four days following the antigenic challenge), or were given only a single injection of diazepam (1.0 mg/kg) or vehicle on the fourth day following SRBC inoculation (30 min prior to spleens and blood being taken). An additional group of mice received no injection to ascertain whether the handling and injection procedure itself might have influenced the immune response (22).

Results

The data were analyzed as a 2 × 2 factorial (Drug Treatment × Chronicity) with an outside control (no treatment). ANOVA indicated that PFC scores varied as a function of the Drug Treatment × Chronicity interaction, $F(1, 34) = 16.13$, $p < .01$. Duncan's multiple range tests indicated that mice repeatedly treated with diazepam exhibited higher PFC scores than the mice acutely treated with diazepam or mice that received repeated vehicle injection (see Fig. 3). Acute diazepam did not influence the PFC response relative to vehicle-treated animals. Likewise, Dunnett's tests ($\alpha = .05$), used to compare the various treatment groups to the outside (untreated) control group, indicated that the PFC response in nontreated animals was comparable to that of vehicle-treated animals, indicating that the injection procedure itself did not influence the immune response.

An ANOVA of the antibody titers revealed that titers were increased in diazepam-treated animals, $F(1, 43) = 17.04$, $p < .01$, and were higher in chronically treated animals relative to mice that received acute treatment, $F(1, 43) = 7.64$, p

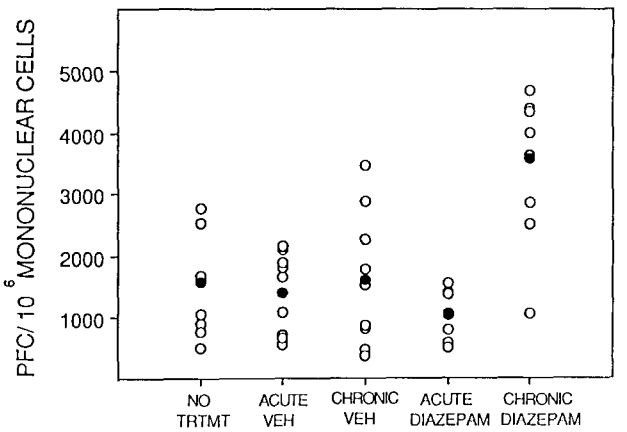

FIG. 3. Individual (○) and the mean (●) splenic plaque-forming cell (PFC) response in mice that had been housed individually for five days prior to sheep red blood cell inoculation and four days afterward and treated with either daily (chronic) or a single (acute) administration of diazepam (1.0 mg/kg) or vehicle. An additional group (NO TRTMT) was housed individually and not otherwise disturbed.

TABLE 3

MEAN (Log_2 + SE) ANTIBODY TITERS AS A FUNCTION OF DRUG TREATMENT AND REGIMEN AMONG MICE INDIVIDUALLY HOUSED FOR FIVE DAYS

Drug Treatment	Mean Antibody Titer
No Treatment	2.77 ± 0.28
Acute Vehicle	2.50 ± 0.31
Chronic Vehicle	2.67 ± 0.28
Acute Diazepam	3.22 ± 0.29
Chronic Diazepam	4.54 ± 0.35*

*$p < .05$ relative to untreated, vehicle, and acute diazepam-treated mice.

< .01. Once again, since an a priori prediction was made concerning the Drug Treatment × Chronicity interaction, Newman-Keuls ($\alpha = .05$) post hoc comparisons were conducted even though this interaction did not reach statistical significance. These comparisons confirmed that chronic treatment with diazepam resulted in higher antibody titers than acute drug treatment or chronic vehicle administration (see Table 3). In contrast, the antibody titers in acutely and repeatedly treated vehicle animals did not differ. Additionally, vehicle treatment did not influence antibody titers relative to nontreated animals.

DISCUSSION

As indicated earlier, several investigators reported that animals housed in groups exhibited a lower immune response than individually housed animals. Such effects were evident with respect to the PFC response, the proliferative response to mitogens, as well as NK cell activity (13,24,25). The results of the present investigation, in contrast, indicated that individual housing was associated with a reduction of PFC response and antibody titers, and that this effect was dependent upon the duration of individual housing. Mice that had been individually housed for 5–10 days prior to SRBC inoculation (i.e., for 9–14 days prior to the peak immune response) exhibited an immunosuppression; however, the suppression was not apparent if mice had been individually housed for 20–30 days prior to inoculation. These findings should not be misconstrued as necessarily being inconsistent with earlier reports. It is not unlikely that the suppression of the PFC response observed in the present investigation was attributable to the change in housing condition coupled with the subsequent individual housing. Specifically, alterations of the PFC response were not observed in mice that had been group housed prior to inoculation, but maintained individually during the four days prior to spleens being taken. Thus these data suggest that the change in housing conditions alone was not sufficient to induce the immunosuppression. It is possible that for a suppression to be provoked the change in housing condition needs to occur some time prior to SRBC inoculation, particularly in light of the finding that the timing of a stressor and SRBC inoculation is fundamental to whether a suppression will appear (32). Alternatively, the effects observed may have been dependent on a change of social environment coupled with some duration of individual housing. Inasmuch as the present investigation was not designed to assess the relative contribution of individual housing and change in housing conditions, it cannot be determined whether a synergistic effect of the two treatments occurred. Whatever the case, it is clear that with continued individual housing beyond 15 days the immunosuppression disappears. Whether such an outcome is a reflection of adaptation to the continued social isolation or a diminution of the stress initially associated with the altered housing remains to be determined.

The notion that change in housing condition is responsible for the altered immune status has been extensively examined. Attempts to dissociate the effects attributable purely to individual housing from those due to change in social mileau generally indicated that the change in the social environment was responsible for the altered immune response (24). The finding that individual housing may lead to enhanced immune functioning, rather than the suppression observed in the present investigation, may stem from the nature of the procedures employed. For instance, in studies where enhanced immune activity was observed following isolated housing, assignment of mice to the individual or group housing condition commenced at weaning (30), or as soon as animals arrived from the breeders (25). Presumably, such procedures precluded the appearance of the immunosuppressive effects that might have been engendered by transferring animals from a colony to individual housing conditions. Indeed, these investigators may have used precisely these procedures to avoid the confounding of the two psychosocial events. Paralleling the immunosuppressive effect of individual housing observed in the present investigation, such a treatment has been reported to increase the growth of transplanted tumors, and this effect was attributable to the change of housing condition. Indeed, it appeared that transfer from isolation to grouping, as well as from grouped to isolated housing, effectively enhanced tumor growth (11,28,31). Of course, these studies do not necessarily imply a role for immune changes in these effects, since other factors, including neuroendocrine or neurotransmitter variations, may have contributed to the alterations of tumor growth (1).

Paralleling the effects of anxiolytics on stressor-provoked central catecholamine changes (14,15,19), treatment with diazepam was found to eliminate the immunosuppression associated with individual housing. Moreover, it appeared that this effect was evident only after repeated treatment of the drug. Of course, it is not known whether a single injection of diazepam at some other time following inoculation might have influenced immune activity. To be sure, it is known that stressors, as well as various manipulations which affect catecholamine activity, may differentially affect immune activity, depending upon the timing of their administration relative to antigen treatment (26,33). Thus, it is conceivable that the impact of anxiolytics might likewise have varied across different aspects of the developing immune response. The fact that diazepam treatment did not influence the PFC response in grouped animals suggests that the impact of the drug was not one of simply enhancing overall immune functioning. Rather, these data suggest that the anxiolytic treatment acted to eliminate the immunosuppression engendered by the individual housing experience. It will be recalled that some stressors have been shown to induce an immunosuppression (2), and treatment with a benzodiazepine inverse agonist induces an immunosuppressive effect reminiscent of that induced by stressor exposure (3,4). Moreover, treatment with anxiolytics has been shown to antagonize the suppression of cell proliferation engendered by surgical stress (12) or foot shock (7). In a like fashion, diazepam treatment in the present investigation may have reduced the stress, anxiety, or arousal associated with changes in housing condition and/or subsequent individual

housing, thereby antagonizing the immunosuppression otherwise observed. Yet it should be underscored that the change in housing condition coupled with subsequent isolated housing does not lead to a profile of physiological changes entirely congruent with that engendered by physical stressors. For instance, unpublished data collected in this laboratory did not reveal this treatment to provoke alterations of plasma corticosterone or variations of norepinephrine and dopamine in either hypothalamic or mesocorticolimbic structures. In this sense it may be inappropriate to describe the housing manipulation as a stressor. In addition, these data suggest that the effects of the housing manipulations on immune functioning were not related to adrenal corticoids, although a role for other aspects of the hypothalamic-pituitary-adrenal axis (e.g., ACTH or corticotropin-releasing hormone, or other peptides) in determining the effects of the housing manipulation or the effects of diazepam cannot be dismissed.

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