16. R. M. Snider *et al.*, in preparation.
17. M. J. Berridge, in *The Neurosciences Fourth Study Program*, F. O. Schmitt and F. G. Worden, Eds. (MIT Press, Cambridge, Mass., 1979), p. 873.
18. E. El-Fakahany and E. Richelson, *J. Neurochem.* **35**, 941 (1980). In brief, intact cells were washed free of growth medium, harvested for assay and suspended in 2 ml of phosphate-buffered saline (PBS) solution. They were then incubated with [^{3}H]guanine (10 μCi/ml) and rotated at 37°C for 45 minutes at 60 rev/min. The cells were briefly centrifuged, the radioactive supernatant was discarded, and then the cells were resuspended in either normal PBS or Ca^{2+}-free PBS and distributed into wells of a multiwell tray in 270-μl portions containing approximately 1×10^5 cells. After incubation of cells at 37°C for 15 to 20 minutes in the shaker bath, at 80 rev/min, thrombin (3 U/ml) was added in a 30-μl volume for a specified time. Basal concentrations of tritiated cyclic GMP were determined by adding 30 μl of PBS. The reaction was terminated by the addition of 30 μl of 50 percent (weight to volume) trichloroacetic acid. After adding to each well 0.6 nCi (1400 dis/min) of ^{14}C-labeled cyclic GMP as an internal standard, the contents of each well were applied to an AG50 W-X2 ion exchange column (0.8 by 8.0 cm) which had been equilibrated with 0.1*N* HCl. The columns were then washed with 4.4 ml of 0.1*N* HCl (eluate discarded) and 1.0 ml of H_2O (eluate discarded), and finally with 1.5 ml of H_2O which was collected in plastic Microfuge tubes. To this eluate, equal (30 μl) volumes of 2.67*M* $ZnSO_4$ and 2.67*M* Na_2CO_3 were added to precipitate any residual [^{3}H]GTP or [^{3}H]GDP. The tubes were then vortexed and centrifuged in a Beckman Microfuge for 2 minutes. The supernatant was transferred to a scintillation vial containing 7 ml of scintillation cocktail and the radioactivity determined in a Searle Isocap/300 liquid scintillation counter. All samples were corrected for the recovery of ^{14}C-labeled cyclic GMP (70 to 80 percent) and for quenching by using the external standard ratio technique.
19. Supported by the Mayo Foundation and PHS grants HL07111 and MH27692.

18 January 1983

Coping and Immunosuppression: Inescapable but Not Escapable Shock Suppresses Lymphocyte Proliferation

Abstract. *Rats were given series of escapable shocks, identical inescapable shocks, or no shock. The subjects were reexposed to a small amount of shock 24 hours later, after which an in vitro measure of the cellular immune response was examined. Lymphocyte proliferation in response to the mitogens phytohemagglutinin and concanavalin A was suppressed in the inescapable shock group but not in the escapable shock group. This suggests that the controllability of stressors is critical in modulating immune functioning.*

Exposure to a variety of psychosocial and environmental stressors can alter the functioning of the immune system (*1*). For example, in humans, lymphocyte proliferation in response to mitogens is suppressed 6 weeks after the death of a spouse (*2*). Such suppression is also seen after exposure to loud noise in mice (*3*), electric shock in rats (*4*), infant-mother separation in bonnet monkeys (*5*), and peer separation in pig-tailed monkeys (*6*). Similarly, acceleration, restraints, and overcrowding in mice reduce the plaque-forming response to sheep red blood cells (*7*).

These stressors are diverse, and it is not known which aspect is critical for the impairment of immune functioning. Research on the relation between stress and cancer suggests that an organism's inability to exert control over the events in question may be important. Psychological states that involve a loss of control, such as bereavement and severe depression, are associated with an increased incidence of cancer (*8*). In fact, an inability to cope with aversive events is frequently part of the definition of stress. It is often argued that an event will induce stress only if the organism cannot or anticipates that it cannot cope with the event (*9*). Stressors that have been shown to impair immune functioning in vitro, such as noise, electric shock, and separation, have been uncontrollable (inescapable and unavoidable).

In a number of experiments the impact of the psychological dimension of controllability on tumor growth and tumor rejection has been evaluated. Sklar and Anisman (*10*) injected mice with syngeneic P815 tumor cells and then gave them 60 shocks. Some of the mice were allowed to terminate each shock by performing an escape response, and thus had a degree of control over the shock. Other mice were given inescapable shocks, and thus had no control. Even though both groups received identical shocks in physical terms, tumor growth was enhanced in the inescapable shock group while the escapable shocks had no effect. Visintainer *et al.* (*11*) reported similar results for the rejection of Walker 256 sarcomas in rats.

However, tumor growth and regression do not necessarily reflect immune system functioning. They can be directly affected by such factors as vascular flow, steroids, and prolactin, all of which are increased by stress (*8*). The purpose of this study was to directly determine whether the controllability of stressors is important in modulating the activity of the immune system. We compared the effects of equal amounts and distributions of escapable and inescapable shock on mitogen-induced proliferation of lymphocytes in vitro. In order to enhance comparability with the tumor studies described above, we used only one session of shocks with qualities similar to those used in the tumor studies. Previous studies of the effects of stressors on in vitro measures of immune functioning have tended to involve multiple sessions of stressor exposure, much more prolonged sessions, or, where shock was used, much more intense shock.

It is not obvious how soon immune functioning should be measured after exposure to shocks of differing controllability. The immediate effects of shock could easily mask any differences between escapable and inescapable shock soon after the session. Suppression could simply be maximum. Indeed, Keller *et al.* (*4*) found no differences in lymphocyte proliferation after shocks of very different intensities when blood was drawn immediately after the session. Suppression was maximum in both high and low shock conditions. In addition, many of the behavioral effects of inescapable shock are typically measured 24 hours after shock exposure rather than soon after exposure. For example, Jackson *et al.* (*12*) reported that inescapably shocked subjects became analgesic 24 hours later on reexposure to a small amount of shock (itself insufficient to produce analgesia). Subjects for whom the initial shocks were escapable did not become analgesic when given shock 24 hours later. However, both escapably and inescapably shocked subjects are analgesic immediately after shock (*13*). Shavit *et al.* (*14*) found footshock that produces an opioid form of analgesia (reversed by opiate antagonists and cross-tolerant with morphine) to be immunosuppressive, whereas footshock that produces a nonopioid form of analgesia was not. The analgesia that emerges upon a brief reexposure to shock 24 hours after inescapable but not escapable shock is completely reversed by opiate antagonists and completely cross-tolerant with morphine (*15*). Thus we measured immune functioning immediately after a brief reexposure to shock 24 hours after experience with equal amounts of escapable or inescapable shock. The procedures used were identical to those found to produce opioid analgesia.

Twelve rats were given an average of one escapable shock per minute, for a total of 80 shocks. Shock intensity began at 0.8 mA and was increased 0.2 mA every 20 trials. Final intensity was thus 1.6 mA. The rats were placed in a small "wheel-turn" box (*16*) and shock was

applied through fixed tail electrodes. Each shock ended when the subject turned the wheel in the front of the chamber. A second group of 12 rats received inescapable shock. Each was paired with an escapable shock subject; shocks began at the same time as for the escapable shock subject and ended when the latter responded. A third group (N = 8) was restrained in the apparatus for an equivalent period of time but was not shocked.

Twenty-four hours later all three groups were given five 5-second, 0.6-mA footshocks in a shuttle box (*16*). Blood was then collected in heparinized Vacutainer tubes by heart puncture under light ether anesthesia. A home cage control group (N = 8) received no experimental treatment before heart puncture. Blood samples were centrifuged for 20 minutes at 400*g* and the plasma was removed. The plasma-depleted whole blood was reconstituted to its original volume in sterile Dulbecco's balanced salt solution.

Lymphocytes were separated under sterile conditions by Ficoll-Hypaque sedimentation and adjusted to a final concentration of 1×10^6 mononuclear cells per milliliter of RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 2 m*M* L-glutamine, and 5 percent heat-inactivated fetal calf serum. White blood cells (before separation) and recovered mononuclear cells were counted with a Coulter counter. The number of recovered mononuclear cells ranged from 4.9×10^6 to 23.6×10^6 per sample. The T-cell mitogens concanavalin A (Con A) and phytohemagglutinin (PHA) were used to stimulate lymphocyte proliferation. Cells (1×10^5) in supplemented RPMI 1640 were added in triplicate to wells of a microtiter plate for each concentration of the mitogens tested and to control cultures to which no mitogen was added. After being incubated for 3 days at 37°C in a humidified incubator with 5 percent CO_2, the cultures were briefly exposed to [*methyl*-^{3}H]thymidine and harvested onto glass fiber filter paper 4 hours later. The incorporation of [^{3}H]thymidine into newly synthesized DNA in stimulated and unstimulated cultures was determined with a liquid scintillation counter.

White cell counts did not differ among groups (*17*). Thus the lymphocytopenia sometimes observed immediately after severe stress (*4*) did not occur. This may be attributable to the fact that only mild shock was received immediately before blood sampling. The initial stressful shock exposure preceded sampling by 24 hours. Figure 1A shows the data on lymphocyte proliferation for the various concentrations of PHA used. At 5.0 μg/ml PHA produced maximum stimulation in all groups. Neither exposure to escapable shock on day 1 followed by five footshocks on day 2 nor restraint on day 1 followed by footshock on day 2 affected lymphocyte proliferation. However, inescapable shock was associated with suppression of lymphocyte proliferation. Analyses of variance yielded reliable differences between groups [$F(3,36) = 4.28$, $P < 0.01$] and among PHA concentrations [$F(3, 108) = 29.86$, $P < 0.001$]. Newman-Keuls post hoc comparisons indicated that the inescapable shock group differed reliably from all the other groups ($P < 0.05$), which did not differ among themselves.

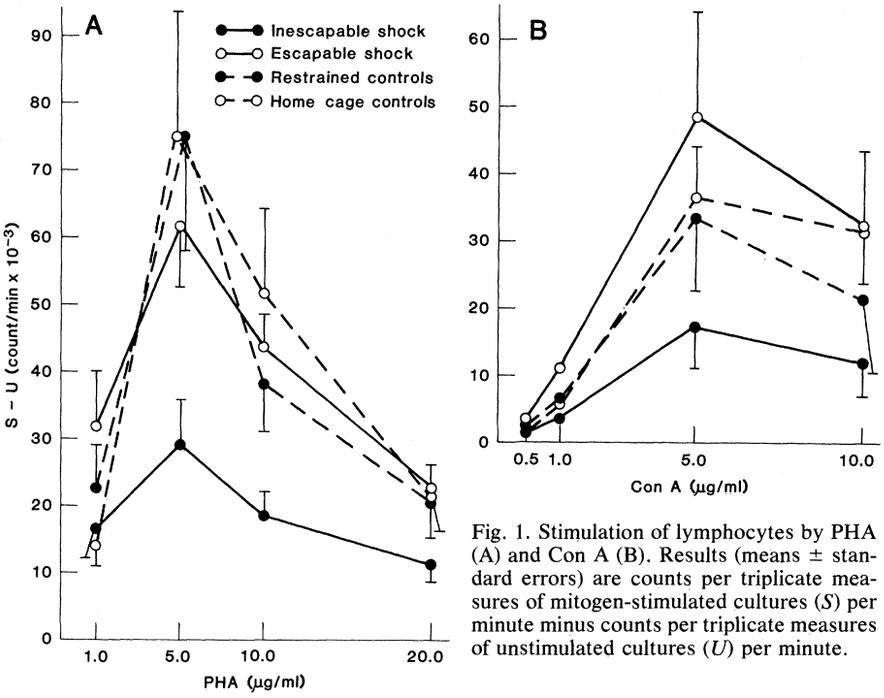

Fig. 1. Stimulation of lymphocytes by PHA (A) and Con A (B). Results (means ± standard errors) are counts per triplicate measures of mitogen-stimulated cultures (*S*) per minute minus counts per triplicate measures of unstimulated cultures (*U*) per minute.

The results for the Con A–stimulated cultures were slightly different (Fig. 1B). This was not unexpected, since Con A and PHA may affect different T-cell subpopulations (*18*). Escapable shock appears to have somewhat facilitated lymphocyte proliferation relative to the restrained control condition. However, inescapable shock again depressed lymphocyte proliferation. The 0.5 and 1.0 μg/ml concentrations did not produce reliable stimulation in control subjects. An analysis of variance was thus applied to only the data obtained for Con A at 5.0 and 10.0 μg/ml. The effect of groups was marginally reliable [$F(3, 36) = 2.49$, $P < 0.07$]. Newman-Keuls comparisons ($P < 0.05$) showed the inescapable shock group to differ from both the escapable shock group and the home cage controls. In addition, the escapable shock group differed from the restrained controls but not the home cage controls.

Thus a single session of 80 brief shocks (shock duration was only 1.0 to 3.0 seconds by the end of training) of moderate intensity can substantially inhibit lymphocyte proliferation in vitro if the subject has no control over the shocks. However, identical shocks produce no decrease in proliferation if the subject can escape them. Thus the ability to exert control over the stressor completely prevented immunosuppression.

We do not know how T cell function was altered by the uncontrollable stress. The T cells could have undergone cellular alteration (*19*), or recirculation patterns could have been changed so that a different subpopulation was represented in the circulation (*20*). Different functional subpopulations would result in a different lymphocyte response. Thus there could have been either a direct functional impairment of lymphocytes or a redistribution of subpopulations producing a change in reactive lymphocytes. Finally, indirect effects on other cell populations, such as the macrophages, could also be responsible for the suppression (*21*).

There are several possibilities for the mechanism whereby inescapable shock led to the change in immune functioning. Adrenal corticosteroids can suppress a variety of immune system functions, including lymphocyte proliferation in response to T-cell mitogens (*3*). Weiss *et al.* (*22*) reported that uncontrollable

stressors produce higher levels of circulating corticosteroids than controllable stressors (*22*). However, since they used stress conditions very different from ours, it cannot be assumed that our groups differed in corticosterone levels. Another possibility concerns endogenous opioids. Opiate antagonists can inhibit tumor growth and prolong survival in animals implanted with tumors (*23*). As already noted, Shavit *et al.* (*14*) found conditions that produce opioid analgesia to be immunosuppressive, while conditions that produce nonopioid analgesia were not. Lymphocytes and neutrophils have been reported to possess opiate receptors (*24*), and our inescapable shock conditions produce opioid analgesia (*15*). Perhaps an endogenous opiate is released, causing immunosuppression. However, many other hormones and neuroregulators affect lymphocyte activity, and many are altered by stress (*25*).

Regardless of the mechanism involved, these findings suggest a link between psychological factors and disease. Psychosocial and environmental factors have long been recognized as affecting health and disease (*1*), and the immune system has often been thought to be the link. However, in experiments involving direct measures of immune functioning, the impact of simple physical stressors has typically been studied. By demonstrating the responsiveness of the cellular arm of the immune system to the dimension of controllability rather than to shock per se, our findings suggest that the immune system can be modulated by more complex psychological factors. These results also suggest that the immune system might be altered by the sorts of variables known to modulate previously studied consequences of uncontrollability, such as learned helplessness (*26*).

MARK L. LAUDENSLAGER*
Department of Psychology,
University of Denver,
Denver, Colorado 80208
SUSAN M. RYAN
ROBERT C. DRUGAN
RICHARD L. HYSON
STEVEN F. MAIER
Department of Psychology,
University of Colorado, Boulder 80309

References and Notes

1. J. G. Rabkin and E. L. Struening, *Science* **194**, 1013 (1976); V. Riley, *ibid.* **212**, 1100 (1981); L. S. Sklar and H. Anisman, *Psychol. Bull.* **89**, 369 (1981); G. F. Solomon and A. A. Amkraut, *Annu. Rev. Microbiol.* **35**, 155 (1981).
2. R. W. Bartrup, L. Lazarus, E. Luckhurst, L. G. Kiloh, R. Penny, *Lancet* **1977-I**, 834 (1977).
3. A. A. Monjan and M. I. Collector, *Science* **196**, 307 (1977).
4. S. E. Keller, J. M. Weiss, S. J. Schleifer, N. E. Miller, M. Stein, *ibid.* **213**, 1397 (1981).
5. M. L. Laudenslager, M. Reite, R. J. Harbeck, *Behav. Neural Biol.* **136**, 40 (1982).
6. M. Reite, R. Harbeck, A. Hoffman, *Life Sci.* **29**, 1133 (1981).
7. R. H. Gisler, *Psychother. Psychosom.* **23**, 197 (1974).
8. B. H. Fox, in *Psychoneuroimmunology*, R. Ader, Ed. (Academic Press, New York, 1981), pp. 103–157; R. B. Shekelle *et al.*, *Psychosom. Med.* **43**, 117 (1981).
9. S. M. Plaut and S. B. Friedman, in *Psychoneuroimmunology*, R. Ader, Ed. (Academic Press, New York, 1981), pp. 3–29.
10. L. S. Sklar and H. Anisman, *Science* **205**, 513 (1979).
11. M. A. Visintainer, J. R. Volpicelli, M. E. P. Seligman, *ibid.* **216**, 437 (1982).
12. R. L. Jackson, S. F. Maier, D. J. Coon, *ibid.* **206**, 91 (1979).
13. S. F. Maier, R. C. Drugan, J. W. Grau, *Pain* **12**, 47 (1982).
14. Y. Shavit, J. W. Lewis, G. W. Terman, R. P. Gale, J. C. Liebeskind, *Soc. Neurosci. Abstr.* **8**, 71 (1982).
15. R. C. Drugan, J. W. Grau, S. F. Maier, J. Madden, J. D. Barchas, *Pharmacol. Biochem. Behav.* **14**, 677 (1981); S. F. Maier *et al.*, *J. Comp. Physiol. Psychol.* **94**, 1172 (1980).
16. S. F. Maier and R. L. Jackson, *Anim. Learn. Behav.* **5**, 407 (1977).
17. Mean white cell counts (± standard deviations) were 15.67 ± 2.82, 16.76 ± 4.07, 13.14 ± 2.62, and 13.99 ± 3.01 cells per cubic millimeter ($\times 10^3$) for the home cage controls, restrained controls, inescapable shock group, and escapable shock group, respectively.
18. E. Nakayama, W. Dippold, H. Shiku, H. F. Oettgen, L. J. Old, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2890 (1980).
19. J. F. Hofert and A. White, *Endocrinology* **82**, 767 (1968).
20. M. M. Zatz, in *Lymphocytes and Their Cell Membranes*, M. Schlesinger, Ed. (Academic Press, New York, 1976), pp. 140–147.
21. J. S. Goodwin and D. R. Webb, *Clin. Immunol. Immunopathol.* **18**, 22 (1981).
22. J. M. Weiss, E. A. Stone, N. Harrell, *J. Comp. Physiol. Psychol.* **72**, 153 (1970).
23. C. F. Aylsworth, C. A. Hodson, J. Meites, *Proc. Soc. Exp. Biol. Med.* **161**, 18 (1979); I. S. Zagon and P. J. McLaughlin, *Life Sci.* **28**, 1095 (1981).
24. E. Hazum, K.-J. Chang, P. Cuatrecasas, *Science* **205**, 1033 (1979); H. W. McCain, I. B. Lamster, J. M. Bozzone, J. T. Grbic, *Life Sci.* **31**, 1619 (1982); A. Lopker, L. G. Abood, W. Hoss, F. J. Lionetti, *Biochem. Pharmacol.* **29**, 1361 (1980).
25. B. R. Newman, V. S. Byers, A. S. Levin, D. F. German, *J. Clin. Lab. Immunol.* **2**, 151 (1979); R. K. Saxena and G. R. Tolivar, *Nature (London)* **268**, 57 (1977).
26. S. F. Maier and M. E. P. Seligman, *J. Exp. Psychol. Gen.* **105**, 3 (1976).
27. Supported in part by NSF grants BNS 78-00508 and RSDA MH00314 to S.F.M. The authors thank R. Harbeck for the use of his laboratory at National Jewish Hospital, Denver. We also thank J. Cohen for his helpful advice.
* To whom correspondence should be addressed.

27 April 1983

Population Density of Tropical Forest Frogs: Relation to Retreat Sites

Abstract. *The forest frog* Eleutherodactylus coqui *defends specific sites for retreats and nests in the Luquillo Forest, Puerto Rico. The hypothesis that shortages of nest and retreat sites limit population size was tested by placing 100 bamboo frog houses in plots measuring 100 square meters in areas of high frog density. These new sites were readily adopted by adult frogs. After one year, experimental plots had significantly more nests and frogs of all sizes than did control plots.*

Shortages in retreat and nest sites are often cited as limits to population size (*1*), but few studies (most of them of birds) have shown the effects on population of an increase in the number of retreat and nest sites (*2*). In studies of reef fishes, critical experiments were either lacking or showed equivocal results (*3*). We now report that population density increases significantly when additional retreat and nest sites are provided for *Eleutherodactylus coqui* Thomas, a terrestrial-breeding frog that is abundant throughout Puerto Rico (*4*). We have studied the biology of *E. coqui* in the Luquillo Division, Caribbean National Forest, since 1978. The coqui is nocturnal, calling and foraging from ground to subcanopy heights, with most activity occurring within 3 m of the ground. Males are territorial and call from exposed sites on, for example, leaves, tree trunks, or human artifacts. Eggs, attended by males, are laid in specific kinds of sites, usually rolled leaves or leaf axils on or near the ground. Of 31 nests located in July 1978, 97 percent were within 1 m of the ground and 87 percent were in rolled leaves on the ground. Such sites also serve as diurnal retreats. At 350 m in mid-elevation forests at El Verde Field Station, where we conducted our study, the preferred nest and diurnal retreat sites are within fallen rolled leaves of *Cecropia peltata* or of the sierra palm, *Prestoea montana*. Frogs are discriminating in their selection of nests and retreat sites (*5*). Our knowledge is based on the location of hundreds of nests and retreats during numerous complete searches of possible off-ground sites as well as ground litter.

Population densities of coqui vary greatly; we have found 1 to 24 adults and 1 to 230 frogs of all size classes per 100 m^2. The highest densities occur in areas of high density of sierra palm. Palms provide axils of leaf bases and fallen fronds, which are favorable calling, foraging, retreat, and nest sites. High population densities, the rapid decay of dried leaves, and the behavior of the frogs suggest that the availability of retreat and nest sites is important in limiting population size. Adult males are territorial and actively defend eggs on which