Toxicant-Disease-Environment Interactions Associated with Suppression of Immune System, Growth, and Reproduction

Abstract. *The effects of marginal malnourishment, infections, and environmental chemicals on growth and reproductive success in Swiss-Webster white mice and wild deer mice were studied with fractional factorial designs. Interaction effects were discovered. For example, malnourished mice were more sensitive to virus exposure and environmental chemicals (a plant growth regulator or polychlorinated biphenyls). Since several commercial plant growth regulators also appear to suppress the immune system, these results cast doubt on the adequacy of current toxicity testing procedures in which factors are studied individually and not in combination.*

Many biological and physical variables affecting an animal's capacity to grow and reproduce have been identified (*1*). Efficient study and modeling of large numbers of variables simultaneously in a biological system pose difficult problems. A multivariable heat and mass transfer model suggests important links between available food and water, climate, and infection-toxicant interactions (*2*). To test the model, we used fractional factorial designs iteratively (*3*) to simultaneously determine main effects and interactions of five variables on growth and reproductive potential in outbred Swiss-Webster white mice and wild deer mice. The variables were availability of food and water, an immunosuppressant chemical (cyclophosphamide, CP) or a plant growth regulator (chlorocholine chloride, CCC), presence or absence of a viral challenge (Venezuelan equine encephalitis, VEE), and fresh wheat sprouts or an environmental contaminant [Aroclor 1254, a mix of polychlorinated biphenyls (PCB's)]. We found that all variables studied except wheat sprouts had significant effects on growth individually or in combination.

When animals have limited resources to fight infection, less mass and energy are available for growth and reproduction (*2*) and for obtaining energy and nutrients. Immunotoxic compounds may interfere with gestation by modifying naturally occurring immunosuppression between mothers and fetuses, increasing susceptibility to infections, and raising maintenance costs through prolonged fevers during infection or disease. PCB's are widespread environmental contaminants (*4*) that may affect animals' immune systems or reproduction (*5*). CCC, which belongs to a group of compounds expected to eventually dominate the agricultural chemicals market (*6*), has immunomodulatory effects in white mice and deer mice (*7*). CP is a known immunosuppressant used to suppress rejection of organ transplants. VEE virus is known to naturally infect rodents, including deer mice (*8*). Vaccine strain TC-83 was used because it infected the mice consistently with minimum mortality.

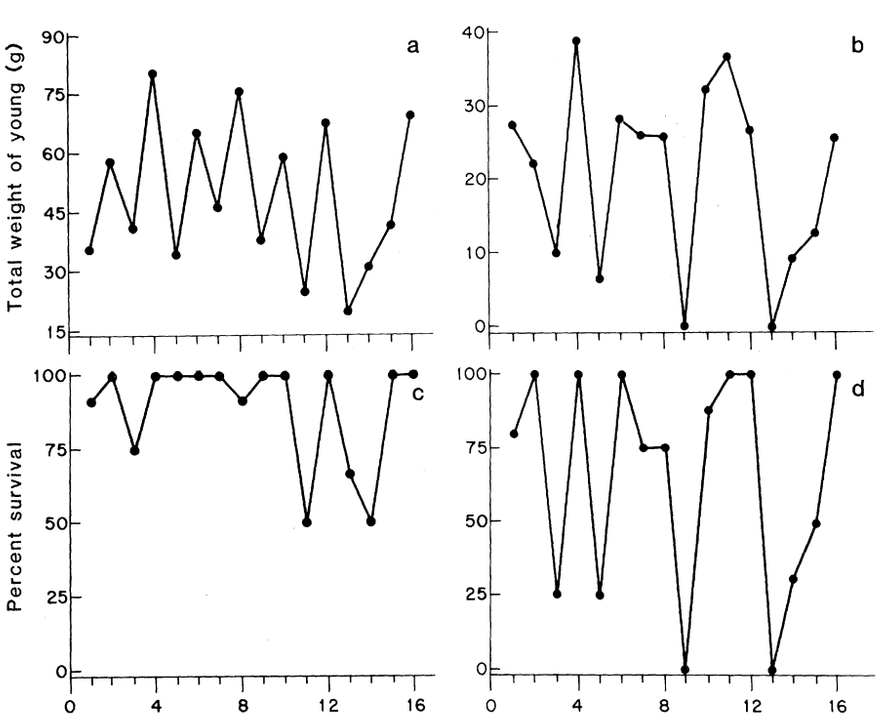

Fig. 1. Average results of experiment 1 on white mice (a and c) and experiment 2 on deer mice (b and d).

Swiss-Webster white mice (*Mus musculus*) and deer mice (*Peromyscus maniculatus*) were used to compare effects on "laboratory" and "wild" species. The white mice were obtained from Harlan Sprague-Dawley and the deer mice came from our breeding colony of approximately 1000 mice, representing mixed stocks from Wisconsin, Michigan, and Virginia. The colony was augmented annually by introduction of wild deer mice captured in Wisconsin. These wild mice were tested for infectious and parasitic organisms and their offspring were used for outbreeding in the colony. Experiments were done in the Biotron, a controlled environment facility on campus. Rebred mothers raising litters were brought to the Biotron early in their pregnancy. Three to seven days before the mice gave birth, chemicals were added to the certified commercial mouse feed if specified in the randomized sequence of experimental runs. The sequence was determined by shuffling numbered cards several times and then dealing the cards from the top of the deck. Pregnant mothers were brought to the Biotron with their litters to ensure that they would raise a litter there. When the new litters were born, young from the previous litter were removed and killed. Within 24 hours after parturition the mothers were injected with virus or diluent alone as specified by the randomized design. Each mother and her young were weighed every third day. Any remaining food and water was removed, weighed, and replaced with fresh rations. If the mother ate the young before 4 days elapsed, we assumed that she was too traumatized by the changes in daily routine to be included in the experiment, and the run was repeated.

The data were analyzed in two steps. In the first step the average daily growth rates of the young were estimated through the first 21 days or until death by fitting linear growth curves. This was justified because for this time span the growth curves were essentially straight lines. In the second step the estimated growth rates were used as the response variable for a regression (factorial) analysis and a calculation of main effects and interactions (Tables 1 and 2).

Five separate 2^{5-1} fractional factorial

designs were performed, each of which contained 16 distinct sets of factor combinations of five experimental variables (Table 3). Additional center points and controls were used. Design points were replicated. In experiment 1, which was conducted on white mice, the variables were mouse feed, water, CP, VEE virus, and wheat sprouts. A minus sign in Table 3 indicates the low level of an independent variable and a plus sign indicates the high level. Low levels were defined as 80 percent of unrestricted food consumption, 80 percent of unrestricted water consumption, no CP, no VEE virus, and no sprouts. High levels were defined as unrestricted food, water, and sprouts; a dose of VEE virus (3×10^4 plaque-forming units) that produces consistent infection with minimum mortality; and a dose of CP (20 mg/kg per day) just sufficient to obtain consistent immunosuppression as determined by the spleen cell plaque-forming assay (*9*). An average of the high and low level for each variable was used for the center replicate level. Each run was replicated at least twice.

Runs 1 through 12 in experiment 1 caused pronounced increases and decreases in the weight of young at weaning that correspond directly to the amount of food available to the animals (Fig. 1a and Table 1). The increases in weight at weaning from runs 14 to 16 when water or food or both were freely available suggest that these animals were sensitive to infectious and chemical agents when there was a moderate reduction in food and water. Percent survival (Fig. 1b) was low when both virus and chemical were present and food or water or both were limited. Food consumption was highest in run 16, which might indicate some compensation for the stress of infection, possibly aggravated by CP intake.

A second experiment of identical design but different randomized sequence was done on wild deer mice. The results (Fig. 1, c and d) were nearly the same as for white mice, except that survival was lower in deer mice when food was limited. In the presence of both immunosuppressant and virus, animals were not able to wean their young when food and water were limited.

Third and fourth experiments of identical designs were conducted with white mice and deer mice, respectively, but different randomized sequences were used and two substitutions of variables were made. We substituted CCC for CP and PCB's for sprouts. Some animals were given CP as a positive control. The low level for the two new chemicals was defined as none added to the feed. High levels were 40 mg/kg per day for CCC and 50 mg/kg per day for PCB's (*10*). Center replicates were half the high values for each chemical. A fifth experiment involving the other half-fraction of a full factorial design of experiment 4 was conducted with deer mice, which allowed us to calculate all main effects and interactions of experiments 4 and 5 free of confounding (*3*). Run sequences were randomized again.

Results for growth rates are shown in Tables 1 and 2. In experiment 1 (white mice) food, water, and VEE virus had significant main effects. In experiment 2 (deer mice), CP, VEE virus, the interaction of CP and VEE virus, and the interaction between CP and sprouts were significant. In experiment 3 (white mice) food had a significant effect. In experiment 4 (deer mice), food, water, and the interaction between water and CCC were significant for deer mice. The estimated coefficients corresponding to main effects and interactions are shown in Fig. 2a with a reference t distribution obtained from the replicate center points. Significant main and interaction effects lie in the shaded area, which corresponds to values greater than 2 standard errors from the mean zero. Over the range studied, available water (variable 2) had a greater effect than available food (variable 1) in promoting growth. VEE virus (variable 4) and PCB's (variable 5) suppressed growth. We also observed a significant block effect, which we ascribe to the difference between seasons, since the first half-

Table 1. Table of estimated coefficients for experiments 1 and 3 (white mice) and 2, 4, and 5 (deer mice). The average and standard deviation of the center replicates and controls are shown. Sample sizes are in parentheses. Theoretically, each growth rate main effect is confounded with a four-factor interaction (*3*). Typically, four-factor interactions are very small relative to main effects, as we found here. An effect on growth rate of a main effect or interaction is the deviation from the average caused by the change in the level of the variable. For example, in experiment 1, changing from 80 percent to free food consumption increases average growth rate 0.100 g/day. Significance levels were computed from the variances in run replicates. Variables for experiments 1 and 2 were food (1), water (2), CP (3), VEE virus (4), and sprouts (5). In experiments 3, 4, and 5 CCC replaced CP as variable 3 and PCB's replaced sprouts as variable 5.

Experiment	Estimates of coefficients of growth rates (g/day)							
	Variable					Center replicates	Controls	Controls given CP
	1	2	3	4	5			
1	0.100*	0.053*	−0.008	−0.033*	0.008	0.22 ± 0.041 (41)	0.53 ± 0.080 (36)	
2	−0.013	0.016	−0.026*	−0.031*	0.020	0.24 ± 0.053 (32)	0.39 ± 0.045 (24)	
3	0.051*	0.003	0.015	−0.033	−0.011	0.45 ± 0.100 (40)	0.64 ± 0.040 (18)	0.59 ± 0.049 (16)
4	0.045*	0.065*	0.014	−0.021	−0.018	0.27 ± 0.137 (21)	0.37 ± 0.044 (28)	0.33 ± 0.96 (16)
4 + 5	0.037*	0.041*	0.006	−0.027*	−0.041*			

*$P < 0.05$.

Table 2. Estimated coefficients of interactions for experiments 1 and 3 (white mice) and 2, 4, and 5 (deer mice) on growth rate (g/day).

Experiment	Two- plus three-factor interaction effects									
	1 × 2 + 3 × 4 × 5	1 × 3 + 2 × 4 × 5	1 × 4 + 2 × 3 × 5	1 × 5 + 2 × 3 × 4	2 × 3 + 1 × 4 × 5	2 × 4 + 1 × 3 × 5	2 × 5 + 1 × 3 × 4	3 × 4 + 1 × 2 × 5	3 × 5 + 1 × 2 × 4	4 × 5 + 1 × 2 × 3
1	0.011	0.015	−0.017	0.013	0.001	−0.005	−0.010	−0.007	−0.001	0.003
2	0.008	0.012	−0.017	−0.010	−0.008	−0.010	−0.002	−0.021*	−0.029*	−0.015
3	0.012	0.010	−0.009	0.014	0.013	0.005	0.009	0.001	−0.013	0.001
4 + 5†	0.005	0.005	0.012	0.003	0.030*	0.008	0.013	0.002	0.016	0.004

*$P < 0.05$. †Two-factor interactions only are shown. There were no significant interaction effects for experiment 4 alone.

Table 3. Half-fraction factorial design used in experiments 1 through 4. The design for experiment 5 was identical to that of the first four, except that the signs were reversed for variable 5. Variables 3 and 5 were CP and sprouts for experiments 1 and 2; they were CCC and PCB's for experiments 3, 4, and 5.

Variable	Run number															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 (food)	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
2 (water)	−	−	+	+	−	−	+	+	−	−	+	+	−	−	+	+
3 (CP/CCC)	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+
4 (VEE virus)	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
5 (sprouts/PCB's)	+	−	−	+	−	+	+	−	−	+	+	−	+	−	−	+

fraction ($+1 \times 2 \times 3 \times 4 \times 5$) was run in fall and winter and the second half-fraction ($-1 \times 2 \times 3 \times 4 \times 5$) in the spring and summer. CCC (variable 3) alone did not have a significant effect, but we observed a significant interaction effect between CCC and water (2×3). The interpretation of this interaction is aided by Fig. 2b, which is a display of the growth rate data as a function of CCC and water alone. We see that when water is restricted (−), CCC causes a lower growth rate (front face); however, when water is unlimited (+), CCC promotes growth (back face). Similarly, the left and right faces show that water has a positive effect but the magnitude of this effect is markedly different depending on the level of CCC. Note that interaction effects could not have been estimated had we used traditional "one factor at a time" experiments. It is not clear why CCC appears to enhance growth when water is not limited.

Interactions between CCC and PCB's had a pronounced effect on birthing success in the experiments on deer mice. The combination of the two chemicals administered before birth in anticipation of parturition resulted in birthing success only 40 and 76 percent of that of control animals given no chemicals in experiments 4 and 5, respectively. Birthing success was 88 and 75 percent of the control value when CCC alone was administered before birth and 54 and 91 percent when PCB's alone were administered. In the first two experiments CP also substantially reduced reproductive success. We anticipated that CCC would affect birthing success in white mice on the basis of preliminary tests that showed immunosuppression and immunomodulatory effects of several other plant growth regulators (cycloheximide, maleic hydrazide, and glyphosine) (*7, 9, 10*).

In summary, administration of an immunosuppressant and virus to pregnant female white mice and deer mice, in combination with moderate limitation of food and water, resulted in reduced growth or reduced survival of young to weaning. In contrast, when both the immunosuppressant and viral infection were present in addition to unlimited food and water, the two species compensated and brought young to weaning, although deer mice did so at weights substantially below control values. These results are important for the following reasons:

1) Potential toxicity of environmental chemicals is commonly tested on experimental animals having access to unlimited food and water. At certain times of the year animals in nature are subject to food or water stress. Thus certain environmental chemicals interacting with infections in natural populations may have substantial effects on mortality and reproduction when food or water is limited.

2) Wild populations may be more susceptible to environmental contaminants than conventional laboratory animals.

3) Interactions of certain "harmless" chemicals at low levels may prove more deleterious than higher doses of "dangerous" toxicants acting alone.

Our results suggest the possibility of added danger to humans and animals if they are malnourished and exposed to a combination of infectious agents and environmental chemicals. While the possibility of added danger from such exposure has long been suspected, the use of fractional factorial designs offers an opportunity to assess biological impact under more realistic conditions than now used in most toxicity studies.

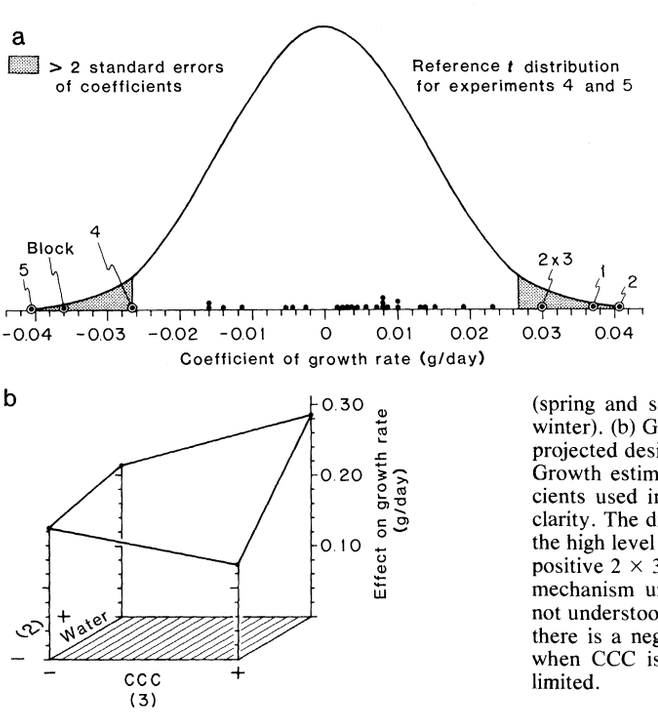

Fig. 2. (a) Plot of deer mouse growth rate coefficients for main effects and interactions for experiments 4 and 5 combined. Significant main effects or interactions are numbered as follows: food (1), water (2), virus (4), PCB's (5), and water and CCC (2×3). *Block* is the block effect due to the time of year the experiments were run (spring and summer versus fall and winter). (b) Growth estimates for the projected design for water and CCC. Growth estimates rather than coefficients used in (a) are presented for clarity. The diagonal from the low to the high level of both variables is the positive 2×3 interaction in (a). The mechanism underlying this effect is not understood. Note, however, that there is a negative effect on growth when CCC is present and water is limited.

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Elevated β-Endorphin in Cerebrospinal Fluid After Electrical Brain Stimulation: Artifact of Contrast Infusion?

Abstract. *β-Endorphin–like immunoreactivity in cerebrospinal fluid was assayed in 11 patients receiving electrical stimulation of the brain for chronic pain. Immunoreactivity increased dramatically after contrast ventriculography prior to stimulation. No further elevations were observed after stimulation. The magnitude and time course of elevations were identical after placement of electrodes either in the thalamus or in the periventricular gray matter. These results suggest that previous findings of stimulation-induced elevation of β-endorphin–like immunoreactivity in cerebrospinal fluid are attributable to an artifact of contrast ventriculography.*

Many studies have shown the effectiveness of electrical stimulation of the periventricular gray matter (PVG) (*1–6*) and various areas of the thalamus or internal capsule (*7–9*) for relief of chronic pain in humans. The most popular neurochemical hypothesis proposed to explain this phenomenon suggests that the effect of stimulation-produced analgesia (SPA) in humans is mediated by release of endogenous opiates. The hypothesis is based on evidence suggesting that (i) analgesia obtained through stimulation of the PVG is effective only in patients whose pain also responds to narcotics (*10*), (ii) intraventricular β-endorphin induces analgesia in patients (*11*), (iii) cross-tolerance occurs between morphine analgesia and SPA (*12, 13*), (iv) naloxone reverses SPA (*14*), and (v) SPA elevates levels of β-endorphin–like immunoreactivity (β-ELI) and methionine-enkephalin–like immunoreactivity in cerebrospinal fluid (CSF). However, several investigators have reported only slight or no reversal of SPA with naloxone administration (*8, 15*), and others have found either no change or inconsistent effects of PVG stimulation on CSF β-ELI (*5, 16*).

We have utilized electrical stimulation of the brain to relieve chronic pain in humans (*17*) and have examined the effects of such stimulation on CSF β-ELI. We report here our neurochemical results.

Four male and seven female patients underwent implantation of stimulating electrodes deep in the brain for a variety of algetic states (Table 1). All patients were completely free of opiate use at the time of the two-stage surgical procedure (*17*). Because some patients with pain of peripheral origin respond to thalamic stimulation as well as PVG stimulation (*9*) and because repeated PVG stimulation can lead to development of tolerance (*1*), all patients receiving a PVG electrode also received a thalamic electrode.

After insertion of a ventricular catheter into the frontal horn of the left lateral ventricle a 2-ml control sample of CSF was obtained. Metrizamide ventriculography was then performed to visualize the third ventricle and Sylvian aqueduct (*18*). In patients receiving a PVG electrode, target coordinates were 1 mm posterior and inferior to the posterior commissure and 1 mm lateral to the wall of the third ventricle. The target for the ventral posterior thalamic (VPT) electrode was determined by the location of the patient's pain. For pain of the extremities or trunk the coordinates were 9 mm posterior, 10 to 12 mm lateral, and 2 to 5 mm dorsal to the midpoint of the anterior commissure–posterior commissure (AC-PC) line. Coordinates for facial pain were 8 mm posterior, 8 mm lateral, and 3 to 5 mm dorsal to midpoint of the AC-PC line. These are the coordinates of Adams and Hosobuchi (*19*). Immediately after insertion of the first electrode (*20*), but before stimulation was begun, a second sample of CSF was obtained. Stimulation was then begun and adjusted to produce maximum relief of pain as reported by the patient. Stimulation parameters arrived at through this method were similar to those in previous studies (*4, 14*). This stimulation was continued for 15 minutes, and a third sample of CSF was obtained. The second electrode was then implanted. Immediately after implantation of the second electrode a fourth sample of CSF was obtained. Stimulation was then begun, adjusted as before, and continued for 15 minutes. A final sample of CSF was obtained after this stimulation. All samples were immediately frozen on dry ice and stored at −90°C until being assayed for β-ELI and β-lipotropin (*21*).

The results of the effects of PVG and VPT stimulation on CSF β-ELI are shown in Fig. 1. Two groups are represented: those patients who received PVG stimulation before VPT stimulation and those patients who received either VPT stimulation before PVG stimulation or who received bilateral VPT stimulation. The latter two (control) groups differ slightly, but both had VPT stimulation first (and thus the first four of five CSF samples were drawn under identical conditions); therefore, the data on them were combined for analysis. Two-way analysis of variance with repeated measures revealed that neither the difference between groups nor the interaction between groups and time was statistically significant. Changes in the level of β-ELI over time (collapsed across groups), however, were significant [$F(4,40) = 13.77$, $P < 0.01$]. Subsequent analysis of this result with a *t*-test with repeated measures demonstrated that β-ELI at each time point significantly exceeded control levels ($P < 0.01$ in each case) (*22*). Therefore, as in previous studies, these results demonstrate significant increases in CSF β-ELI in patients receiving electrical stimulation of the brain for chronic pain (*2–4*).

β-Lipotropin in CSF increased only 50 percent on average after contrast infusion and did not change further with