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Voluntary Consumption of Cyclophosphamide by Nondeprived Mrl-lpr/lpr and Mrl +/+ Mice

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GROTA, L. J., R. ADER, J. A. MOYNIHAN AND N. COHEN. *Voluntary consumption of cyclophosphamide by nondeprived Mrl-lpr/lpr and Mrl +/+ mice.* PHARMACOL BIOCHEM BEHAV **37**(3) 527–530, 1990.—Cyclophosphamide dissolved in several dilutions of chocolate milk was presented for 20 hr daily to nondeprived, symptomatic, autoimmune Mrl-lpr/lpr and asymptomatic Mrl +/+ mice. In the absence of cyclophosphamide, daily consumption was inversely related to the concentration of the chocolate milk solutions and increased from the first to the fourth day of exposure. There were no effects of strain or sex on the consumption of plain chocolate milk. Consumption of 0.1 or 0.2 mg cyclophosphamide per ml of different dilutions of chocolate milk increased over days 1–4 and decreased on day 8. Consumption of 0.4 mg/ml cyclosphosphamide did not change over days. Generally, consumption was inversely related to the cyclophosphamide concentration. Females consumed more cyclophosphamide than males. Autoimmune lpr/lpr mice consumed more cyclophosphamide than +/+ mice. Dilution of chocolate milk had no effect on consumption of cyclophosphamide. Lymphoproliferation and anti-ssDNA antibody titer were reduced by the consumption of cyclophosphamide-chocolate milk solutions. It is hypothesized that autoimmune lpr/lpr mice voluntarily consume more cyclophosphamide than asymptomatic +/+ mice in an effort to "correct" their immune system dysregulation.

Autoimmune disease Drinking behavior Cyclophosphamide Mrl-lpr/lpr mice

IN previous studies, we have found that autoimmune disease-prone mice do not acquire conditioned taste aversions to saccharin or chocolate milk in response to pairings with injected doses of cyclophosphamide (CY) that are effective unconditioned stimuli for normal or asymptomatic mice (1,4). Moreover, we have observed that autoimmune Mrl-lpr/lpr mice consume more CY, an immunosuppressive drug, dissolved in chocolate milk than congenic Mrl +/+ mice that do not have symptoms of autoimmune disease (5). These substrain differences were not observed when lpr/lpr and +/+ animals were tested before the onset of autoimmune symptoms in lpr/lpr animals. These substrain differences were also not observed when the lpr/lpr and +/+ mice were exposed to increasing doses of the nonpreferred, nonimmunomodulating substance quinine hydrochloride dissolved in chocolate milk that reduced consumption to levels comparable to the CY-exposed mice (2). In these studies, chocolate milk (CHOC) solutions were presented to mice adapted to a 23-hr fluid deprivation regimen. Scheduled drinking periods synchronize some but not all circadian rhythms (3, 6, 7, 9). It is not known whether these short drinking periods and altered relationships among circadian rhythms interact to influence consumption of CHOC/CY. Also, during a 1-hr drinking period, the fluid-deprived mouse is forced to drink in order to sustain life. The brief (1 hr) availability of fluid might mask age-related differences in consumption, and the cues associated with drinking behavior resulting from hunger/thirst may mask those from the autoimmune disease. Presumably, the saliency of cues from the presence of autoimmune disease would be higher in nondeprived animals.

It is apparent that CY is unpalatable to mice since they will voluntarily consume only small amounts when it is dissolved in water (unpublished observations from our laboratory). In previous studies, voluntary consumption of CY sufficient to alter the immune status of mice occurred when it was dissolved in highly preferred whole chocolate milk. The extent to which the greater consumption of CHOC/CY by fluid-deprived autoimmune lpr/lpr mice was influenced by the taste of the chocolate milk is not known. Thus, to determine the generality of previous observations (1, 4, 5), consumption of CY in several dilutions of chocolate milk (in water) was investigated in nonfluid-deprived autoimmune lpr/lpr and congenic +/+ mice.

METHOD

A single population of Mrl-lpr/lpr and Mrl +/+ mice born in

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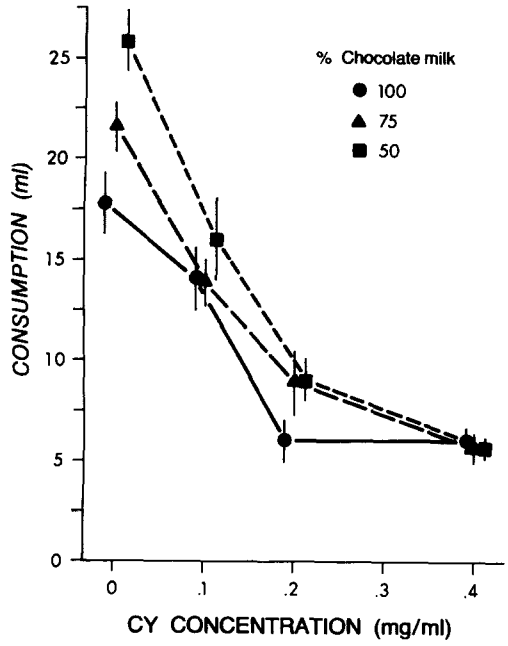

FIG. 1. Effects of chocolate milk dilution and concentration of cyclophosphamide on consumption (ml) of CHOC/CY solutions. Each point represents the mean ± SE of 14–20 animals.

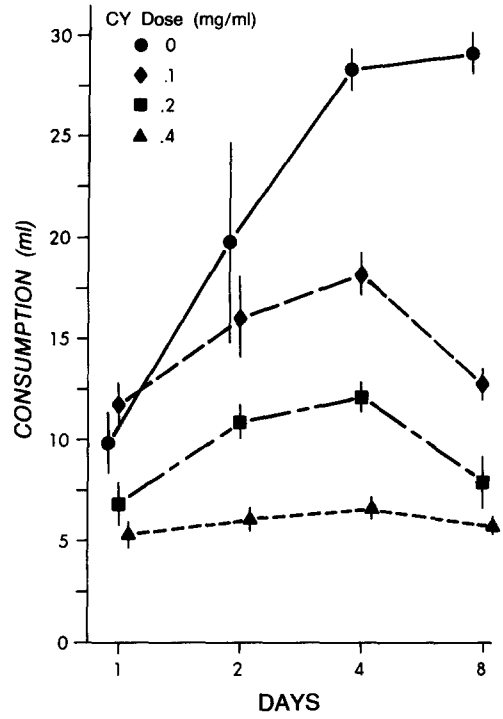

FIG. 2. Comsumption (ml) of chocolate milk containing cyclophosphamide on days 1–8 of exposure. Each dose represents 50–57 animals. Mean ± SE are shown.

our laboratory was housed individually after weaning in a temperature controlled laboratory under a 12-hr light-dark cycle with lights on at 6 a.m. At 18 weeks of age, when most lpr/lpr animals are showing evidence of autoimmune disease (increased titer of antibody against DNA and lymphadenopathy), their water bottle was replaced with a bottle containing 0,0.1, 0.2, or 0.4 mg cyclophosphamide (CY, Sigma Chemical Co, St. Louis, MO)/ml Sealtest whole chocolate milk (100%), 75% chocolate milk diluted in water, or 50% chocolate milk diluted in water. These CHOC/CY drinking solutions were present on the mouse's cage from early afternoon until the next morning when they were removed for 3–4 hours for weighing, washing, refilling, and reweighing. The mice were exposed to fresh CHOC/CY solutions Monday through Friday with ad lib water on the weekends. Consumption was measured after 1, 2, 4 and 8 days of exposure to the chocolate milk solutions. Palpable lymphoproliferation (4) and circulating antisingle-stranded-DNA antibody titers were assayed before and after Days 4 and 8. Blood was obtained by retroorbital bleeding under CO_2 anesthesia and serum was frozen until assayed for ssDNA as described previously (4).

RESULTS

An overall analysis of variance (4 Doses × 3 Dilutions × 2 Sexes × 2 Strains × Days; repeated measures at 4 time points) of CHOC/CY consumption revealed a significant Dose × Dilution interaction, $F(6,163) = 7.99$, $p<0.01$. This interaction, shown in Fig. 1, occurred because dilution of plain chocolate milk (0 CY) resulted in increased consumption whereas in the presence of cyclophosphamide, dilution of chocolate milk had little effect on consumption (see below). The analysis also revealed an interaction between Days and Dose, $F(9,486) = 15.2$, $p<0.01$. The data from this interaction are shown in Fig. 2. On the initial day of exposure, consumption of CHOC/CY was relatively low with consumption of 0.2 and 0.4 mg CY/ml CM less than 0 and 0.1 mg CY/ml CM [0 vs. 0.2: $t(163) = 2.54$, $p<0.01$]. On subsequent days, consumption increased for zero CY animals and less so as the concentration of CY was increased. Consumption of 0.1 and 0.2 mg/ml CHOC/CY increased from Day 1 to Day 4 and decreased to Day 1 levels by Day 8. Animals given 0.4 mg/ml CHOC/CY consumed similar amounts on all 8 days. No other interactions reached significance.

The overall analysis also indicated that lpr/lpr mice (mean = 13.74 ± 0.55 ml, N = 114) drank more than +/+ mice (mean = 12.22 ± 0.49 ml, N = 100), $F(1,163) = 12.7$, $p<0.01$. Because diluting chocolate milk had such a marked increase on the consumption of plain chocolate milk and little effect on the consumption of CY, separate analyses of variance of the consumption of plain and CY-laced chocolate milk were calculated. As noted above (Fig. 1), analysis of plain chocolate milk consumption revealed main effects for Dilution, $F(2,38) = 10.9$, $p<0.01$, and Days, $F(3,114) = 22.5$, $p<0.01$. Consumption of 100% chocolate milk was 17.8 ± 1.5 ml (N = 17), while consumption of 75% chocolate milk was 21.6 ± 1.3 ml (N = 19) and consumption of 50% chocolate milk was 25.9 ± 1.5 ml (N = 14). Across Days, the 50 mice given plain chocolate milk initially consumed 9.9 ± 1.5 ml; consumption increased to 19.7 ± 5.1 ml on the second day and to 28.4 ± 0.95 and 29.1 ± 1.05 ml by the fourth and eighth day of exposure (see Fig. 2). There were no effects of Strain or Sex, nor were there any interactions of these variables with Dilution and Days.

In contrast to these results, analysis of variance of CY consumption revealed that Mrl-lpr/lpr mice consumed more of the CHOC/CY solutions than +/+ mice, $F(1,133) = 8.11$, $p<0.01$. Lpr/lpr mice (N = 86) consumed 10.7 ± 0.6 ml CHOC/CY and +/+ mice (N = 78) consumed 9.4 ± 0.5 ml CHOC/CY. The analysis also indicated that females (11.4 ± 0.7 ml, N = 81) consumed more CHOC/CY than males (8.8 ± 0.4 ml, N = 83;

TABLE 1

SUM OF RANKINGS FOR ANTI-DNA ANTIBODY TITER FOR lpr MICE DRINKING VARIOUS DOSES OF CYCLOPHOSPHAMIDE DISSOLVED IN CHOCOLATE MILK

Cyclophosphamide	Before Treatment	Day 4 Treatment	Day 8 Treatment	N
0	38	67.5	62.5	28
0.1 mg/ml	51	72	45	28
0.2 mg/ml	51	75	48	29
0.4 mg/ml	51.5	78	44.5	29

$F(1,133) = 31.1$, $p<0.01$, but there were no interactions involving gender. The analysis revealed no main effect nor interactions with dilution.

Palpable lymphoproliferation in lpr/lpr mice was analyzed separately for each dose of cyclophosphamide. Among the mice given plain chocolate milk, there was no change in lymphoproliferation during the course of the experiment ($Q = 5.14$, $p<0.10$), but the tendency was for lymphoproliferation to increase. In contrast to this observation, mice given cyclophosphamide had reduced lymphoproliferation (0.1 mg/ml: $Q = 10.2$, $p<0.01$; 0.2 mg/ml: $Q = 12.25$, $p<0.01$; 0.4 mg/ml: $Q = 14.22$, $p<0.001$).

Circulating anti-ssDNA antibody titers are elevated over a broad range in lpr/lpr mice and indicate increasing severity of disease (11). Because of the broad range of titers between animals, titers from individual animals were ranked over the three bleeds (Before treatment, Day 4 of treatment, and Day 8 of treatment) and the chi-square for correlated samples (Friedman χ^2) calculated separately for each CY dose (see Table 1). The analysis indicated that anti-ssDNA titers increased during the experiment for the animals given no CY, $\chi^2(2) = 17.7$, $p<0.01$. The animals given CY had increased titers on Day 4 relative to the titer before treatment but by Day 8 titers were reduced for all doses of cyclophosphamide [0.1: $\chi^2(2) = 14.2$; 0.2: $\chi^2(2) = 15.3$; 0.4: $\chi^2(2) = 21.7$; $p<0.01$].

DISCUSSION

Increased consumption of cyclophosphamide by autoimmune Mrl-lpr/lpr mice compared to congenic +/+ controls was observed when fluid deprived mice were exposed to CY dissolved in chocolate milk for 1 hr/day (5). Since we hypothesized that the increased consumption of CY reflects the therapeutic effects of the immunosuppressive drug for the dysregulated immune system of lpr/lpr mice, we would expect similar findings even though exposure to CY occurred under different conditions. In the current experiment, CHOC/CY solutions were presented to nondeprived animals and we observed that Mrl-lpr/lpr animals that were beginning to manifest symptoms of autoimmune disease consumed more CHOC/CY than asymptomatic Mrl +/+ controls. In earlier stages of the autoimmune disease, lpr/lpr and +/+ animals do not differ in consumption of CY (5).

Mrl-lpr/lpr and +/+ mice do not differ in consumption of plain chocolate milk. It is also of interest that female lpr/lpr mice that exhibit the autoimmune disease earlier and perhaps more intensely than male lpr/lpr mice consume more CY than male lpr/lpr animals. Taken together, these data support the hypothesis that increased consumption of the CHOC/CY solutions reflects a behavioral strategy that modulates immune system function.

One hypothetical explanation for the higher consumption of CHOC/CY solutions by lpr/lpr mice is that they have a need for higher caloric intake than the +/+ mice. Other studies (8,10), however, have shown that most autoimmune mouse strains, including Mrl, live longer and have reduced autoimmune symptoms on caloric restriction. Based on these observations, one would predict that lpr/lpr mice might ingest less chocolate milk than +/+ mice. The fact that they consume more of the CHOC/CY solution suggests that this response is determined more by the therapeutic effects of the cyclophosphamide than by the caloric content of the carrier milk solution (to which they may also be responding).

Consumption of CY in chocolate milk is influenced not only by the consequences of CY but also by the taste of both CY and chocolate milk. Diluting plain chocolate milk with water increased consumption but, even under these conditions, the addition of CY reduced consumption to essentially the same levels independent of the dilution of the chocolate milk. These data indicate that the CY (taste, consequences, etc.) determines the consumption of CHOC/CY rather than the taste of the chocolate milk masking solution.

Lymphoproliferation and anti-DNA antibody titer were assayed to determine whether voluntary consumption of CY by Mrl-lpr/lpr mice would be sufficient to alter either of these two markers of autoimmune disease. Consumption of CY results in a reduction in the number of animals with lymphoproliferation within a few days of the start of CY intake and remains low thereafter. Autoantibody titers are elevated in lpr/lpr animals and the consumption of CY also reduces anti-DNA titer. These results suggest that increased consumption of CY by autoimmune mice is related to an improvement of their disease state. At this stage, the data are only correlational but they are consistent with the hypothesis that mice with autoimmune disease increase their voluntary consumption of cyclophosphamide to reduce the effects of their dysregulated immune system.

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