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Effects of Exogenous Corticosterone Treatment on Alloantigen-Specific Cytotoxic T Lymphocyte Activity in Mice[1]

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ABSTRACT

The intent of this study was to examine the effects of stress-like plasma corticosterone (CS) elevation on the generation of alloantigen-specific cytotoxic T lymphocyte (CTL) activity in mice. Elevation of plasma CS was achieved by infusion of exogenous CS via osmotic pumps. CS infusion at 16 mg/kg/day on days -4 through 10 relative to alloantigen challenge led to slight, but significant, suppression of CTL activity on day 10 but no elevation of plasma CS levels. Infusion of lower CS doses (1, 2, 4 or 8 mg/kg/day) had no effect on CTL activity. Serial sampling of mice infused with CS at 0.09, 0.9 or 9 mg/kg/day over a 14-day period indicated that only the 9 mg/kg/day infusion rate caused significant plasma CS elevation. Peak CS levels (~500 ng/ml) were observed 1 day after the start of CS infusion, but CS levels fell to below 200 ng/ml by day 7 and were ~50 ng/ml on day 12 indicating that elevated plasma CS levels could not be maintained for extended periods by CS infusion. An attempt to define the windows of CS sensitivity during CTL development was made by infusing mice with CS at doses of 10–16 mg/kg/day on days 0–3, 3–6, 4–7, 5–8 and 6–9, relative to alloantigen challenge; however, CS infusion had no effect on CTL activity. In contrast, dexamethasone infusion (9.4 mg/kg/day) on days 0 to 3 suppressed CTL activity by ~90% indicating that the generation of CTL activity is sensitive to high dose GC treatment, but is refractory to stress-like CS elevation. In mixed lymphocyte-tumor cell cultures, CTL activity was suppressed by CS (2.5×10^{-8} M) if added on the first day of culture but not if added on subsequent days. These results suggest that CTL are most sensitive to CS-induced suppression if exposed near to the time of alloantigen challenge.

GC are potent immunosuppressive and antiinflammatory compounds (see reviews by Cupps and Fauci, 1982, and Boumpas *et al.*, 1991, 1993). GC have been used in both human medicine and laboratory animal research. Synthetic rather than natural GC are used most often because of their longer half-lives and greater potency. The immunosuppressive potency of synthetic GC (*e.g.*, dexamethasone) has led to their use as benchmark immunosuppressive compounds in immunotoxicity testing (Exon *et al.*, 1990).

Because of the immunosuppressive potency of exogenously administered GC, enhanced production of endogenous GC and elevation of circulating GC levels has been suspected to induce immune suppression. This is supported by numerous studies that have correlated stress-induced elevation of endogenous GC, such as that induced by shock (Keller *et al.*, 1983), rotation (Riley, 1981) and noise (Monjan and Collector, 1977), with altered immune function (reviewed by Pruett *et al.*, 1993). More recently, immune suppression has been correlated with elevation of circulating GC levels in laboratory animals exposed to immunotoxic xenobiotic compounds, *e.g.*, ethanol (Jerrells *et al.*, 1990), benzene and toluene (Hsieh *et al.*, 1991), morphine (Bryant *et al.*, 1991; Pruett *et al.*, 1992; Sei *et al.*, 1991), gallium arsenide (Burns *et al.*, 1994) and phenytoin (Hirai and Ichikawa, 1991).

In this laboratory, a correlation between endogenous GC elevation and suppression of CTL activity has been investigated in mice exposed to HxCB, an immunotoxic polychlorinated biphenyl isomer. Treatment of mice with HxCB leads to a dose-dependent suppression of splenic CTL activity coincident with a dose-dependent elevation of plasma CS levels (Kerkvliet *et al.*, 1990). In mice treated with HxCB at 10 mg/kg body weight, suppression of CTL activity by ~80% has been correlated with peak plasma CS levels reaching 200 to 550 ng/ml (DeKrey *et al.*, 1993; Kerkvliet *et al.*, 1990). Inasmuch as exogenous GC treatments have been reported to suppress *in vivo* generated CTL activity in mice (Eishi *et al.*, 1983; Freise *et al.*, 1991; Conlon *et al.*, 1985; Kajiwara, 1988; Borel, 1976), elevation of plasma CS levels was suspected as a mechanism of HxCB-induced CTL suppression (Kerkvliet *et al.*, 1990). However, treatment with RU 38486 (a GC receptor antagonist) did not alter the degree of CTL suppres-

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ABBREVIATIONS: CS, corticosterone; CTL, cytotoxic T lymphocyte; GC, glucocorticoid; HxCB, 3,3′,4,4′,5,5′-hexachlorobiphenyl; CTX, cytotoxicity; MLTC, mixed lymphocyte-tumor cell; LU, lytic unit; E:T, effector:target cell.

sion in HxCB-exposed mice (De Krey *et al.*, 1993) suggesting that CS elevation does not play a significant role in HxCB-mediated CTL suppression.

In our study, the ability of exogenous CS to suppress *in vivo*-generated CTL activity was examined as a way to examine the immunosuppressive potency of elevated endogenous CS. Exogenous CS was administered by constant sc. infusion using osmotic pumps. Osmotic pumps were implanted at various times relative to alloantigen challenge to examine the time-dependent sensitivity of CTL to suppression by CS. In addition, for purposes of comparison, the time-dependent sensitivity of *in vitro* generated CTL to CS exposure was examined using MLTC cultures.

Methods

Animals. Male C57Bl/6 mice, 6 wk of age, were obtained from The Jackson Laboratories (Bar Harbor, ME). Animals were housed in polycarbonate shoe-box cages in front of a sterile laminar flow device and acclimated for a minimum of 7 days before experimentation. A maximum of four animals were housed per cage. Cages were randomly assigned to positions in a cage rack, and animals were provided with Bed-O-Cob bedding (The Andersons, Maumee, OH). Food (Wayne Rodent Blox, Harlan Sprague Dawley Co., Bartonville, IL) and tap water were available *ad libitum*. Animal rooms were maintained on a 12-hr light/dark cycle (fluorescent, 7:30 AM lights on) and constant temperature (72 ± 1 [°F]) and 50% humidity. The mice remained free of all common murine pathogens as assessed by Charles River Professional Services, Wilmington, MA.

Chemicals. Dexamethasone sodium phosphate was obtained from Anthony Products (Arcadia, CA) as an aqueous solution at 4 mg/ml and was used without further dilution. For *in vivo* use, CS (Sigma Chemical Co., St. Louis, MO) was dissolved in polyethylene glycol 400 (Sigma). Osmotic pumps (Alza Corp., Palo Alto, CA) were filled with dexamethasone, CS or polyethylene glycol 400 vehicle. For *in vitro* use, CS was dissolved in ethanol and diluted in culture media.

Animal treatment. While mice were under anesthesia (ketamine and xylazine), pockets were formed to the left of the dorsal midline and osmotic pumps were placed into them. Some mice were also adrenalectomized (DeKrey *et al.*, 1993) at the time of pump implantation. Some mice were bled by clipping the end of the tail, and blood was collected into EDTA treated capillary tubes. Plasma was isolated and stored at -20°C until analyzed.

The P815 mastocytoma cell line was propagated in ascites form by weekly passage in syngeneic DBA mice (H-2^d). P815 cells were harvested from DBA mice after 6 to 8 days of *in vivo* growth. C57Bl/6 mice (H-2^b) received an i.p. injection with 1.0×10^7 viable P815 cells in 0.5 ml of Hanks' balanced salt solution. Mice were killed 10 days later at the time of peak CTL activity (Kerkvliet and Baecher-Steppan, 1988). Spleens were removed and single-cell suspensions were prepared as described previously (Kerkvliet and Baecher-Steppan, 1988) in RPMI 1640 (BioWhittaker, Walkersville, MD) containing 5% fetal bovine serum (Rehatuin, Intergen, Purchase, NY), 20 mM HEPES buffer and 5×10^{-5} M 2-mercaptoethanol.

MLTC cultures. The MLTC culture conditions used in these studies were based on the methods of House *et al.* (1989). P815 cells ($P815_m$) were incubated with mitomycin C (Sigma) at 50 μg/ml for 30 min in minimal essential medium (GIBCO, Life Technologies Inc., Grand Island, NY) containing 10% fetal bovine serum, 20 mM HEPES buffer, 50 μg/ml gentamicin, 5×10^{-5} M 2-mercaptoethanol and 2 mM glutamine. $P815_m$ were washed four times before use. Single cell suspensions of spleen cells from C57Bl/6 mice were prepared as described. Spleen cells (1.5×10^7) were cocultured with 3×10^5 $P815_m$ in 25-cm tissue culture flasks or six-well tissue culture plates (Corning, Corning, NY) and 10 ml medium. CS- or ethanol-containing medium was added on various days after culture initiation. The final concentration of ethanol in all cultures was 0.02%. Cultures were incubated in 10% CO_2 at 37°C. After 5 days, the CTL activity of viable cells from each culture was measured. The concentration of viable cells in each culture was determined as follows: 50-μl culture samples were incubated with equal volumes of pronase (5 mg/ml) for 10 min at 37°C. Each sample was then diluted in Isoton (Coulter Electronics, Hialeah, FL) and Zapoglobin (Coulter) and incubated at room temperature for 5 to 10 min. The concentration of cells in each diluted sample was determined using a Coulter Counter.

CTL assay. CTL activity was measured in a 4-hr ^{51}Cr release assay as described previously (Kerkvliet and Baecher-Steppan, 1988; DeKrey *et al.*, 1993). The percent CTX at each effector:tumor cell (E:T) ratio was calculated by the equation:

$$\% \text{ CTX} = \frac{test - naive}{mr - naive} \times 100,$$

where *test* = cpm using P815-stimulated spleen cells, *naive* = cpm using P815-naive spleen cells and mr = the maximum cpm released from cultures incubated with either sodium dodecyl sulfate or Tween 80. Inasmuch as preliminary studies indicated that freshly isolated spleen cells were ≥90% viable (data not shown), the viability of spleen cells was not determined before assay for *in vivo* generated CTL. The viability of cultured cells was determined in each experiment and E:T ratios were calculated based on viable cells. In order to compare the overall cytotoxic potential of animals or MLTC cultures, LU/spleen were calculated using a method adapted from Bryant *et al.* (1992) by the equation:

$$\text{LU/Spleen} = \frac{\text{Total Spleen Cells}}{10^6 \times e^{(\bar{Y}^* - C \times \overline{\ln(x)} - \ln(P/(A-P)))/C)/T}} \times 100,$$

where Y^* = logit transformed percent lysis, C = mean slope of the logit transformed percent lysis curve for each group, x = E:T ratio, A = maximum limiting percent lysis (75%), P = reference lysis (30%), and T = number of target cells. LU are presented per spleen for *in vivo* data and per culture for *in vitro* data.

CS radioimmunoassay. Plasma CS levels were determined using [^{125}I]-coupled double antibody radioimmunoassay kits (ICN Biochemicals, Carson, CA). A protocol optimized by the manufacturer was used. The lower limit of detection was 25 ng CS/ml. The maximum interassay and intraassay coefficients of variance for internal controls were 15.7% and 8.8%, respectively, as determined by the manufacturer. Samples with analyte levels below the lower limit of detection were assigned the limit value of 25 ng/ml.

Statistics. Statistical analyses were performed using the SAS statistical software database (version 6.03, SAS Institute Inc., Cary, NC) for the IBM personal computer. Comparisons of two sample means were performed using *t* tests (TTEST of SAS). Comparisons of more than two sample means were performed using general linear models (GLM of SAS). Unless otherwise indicated, values of $P \leq .05$ were considered statistically significant.

Results

Study 1: The effects of 14 day continuous CS infusion on CTL activity. The intent of this study was to determine if infusion of exogenous CS via osmotic pump would suppress alloantigen-specific CTL activity in mice. Based on CS solubility tests in this laboratory, a CS infusion rate of 16 mg/kg/day was the highest infusion rate that could be achieved using 14 day osmotic pumps (Alzet, model 2002). This infusion rate is approximately 10-fold higher than the rate sufficient to elevate CS to basal levels (~50 ng/ml) in adrenalectomized mice (G. K. DeKrey, unpublished results).

Therefore, 16 mg/kg/day was chosen as the highest infusion rate for this study. Four days before alloantigen challenge, mice were implanted with osmotic pumps designed to deliver CS at 1, 2, 4, 8 or 16 mg/kg/day for 14 days. All mice were killed 10 days after alloantigen challenge.

As shown in figure 1, infusion of CS at 16 mg/kg/day led to a slight, but significant, suppression of CTL activity. In contrast, no significant suppression of CTL activity was observed in mice that received CS at dose rates of 8 mg/kg/day or lower. The total splenic CTL potential, calculated as LU/spleen, was reduced by 41% and 64% in mice infused with CS at 8 and 16 mg/kg/day, respectively, but these effects were not statistically significant (table 1). Interestingly, thymus and spleen weights were significantly affected by CS at lower dose rates than that required to alter CTL activity. As shown in table 1, the minimal dose rate that led to a significant reduction in thymus weight was 2 mg/kg/day, whereas a dose rate of 4 mg/kg/day led to significant loss of spleen weight and cells. Higher CS dose rates led to more pronounced effects in a dose-dependent manner.

At the time of euthanasia, plasma was collected to verify elevated CS levels in CS treated animals. Interestingly, unlike the dose-dependent effects on organ weights, no dose-dependent elevation of plasma CS was observed (table 1). Significant plasma CS elevation was only observed in mice that received CS at a rate of 4 mg/kg/day.

Study 2: Time-dependent elevation of plasma CS levels in mice infused with exogenous CS. The intent of this study was to examine the time-dependent effects of CS infusion on plasma CS levels in mice. In the previous study, a dose-dependent elevation of plasma CS levels was expected in mice receiving continuous CS infusion, but none was observed. However, because only one time point was examined (14 days after the start of CS infusion), it was unclear if the CS levels observed were representative of the actual CS levels that occurred on the previous days. Therefore, in this study, plasma CS levels were examined in CS-treated mice on various days after the start of CS infusion up to day 12. ADX mice were used to permit serial blood sampling (approximately every 3 days by tail vein) without causing elevation of plasma CS levels due to stress-induced endogenous production. CS was administered at dose rates of 0.09, 0.9 or 9.0 mg/kg/day via osmotic pumps that were fitted with a length of tubing that delayed the onset of CS infusion until the day after surgery. In this way, the adrenalectomized status of each mouse (lack of detectable plasma CS levels) could be verified before the start of CS infusion.

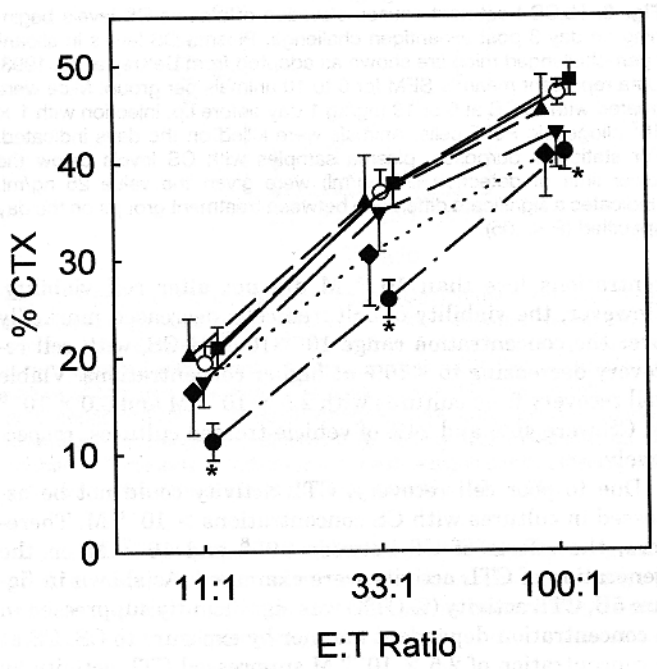

Fig. 1. Effect of CS infusion from day -4 through day 10 relative to alloantigen challenge on CTL activity. CTL activity is presented as % CTX at various E:T ratios (mean ± SEM) for six to eight mice per group. CS-treated animals were implanted with osmotic pumps 4 days before alloantigen challenge. The pumps were designed to release CS at a constant rate [vehicle (○), 1 mg/kg/day (▼), 2 mg/kg/day (■), 4 mg/kg/day (▲), 8 mg/kg/day (♦), 16 mg/kg/day (●)] for 14 days. Animals were killed on day 10 post alloantigen challenge and splenic CTL activity was measured as described in "Methods." *Indicates the mean is significantly different from untreated control (vehicle) at the E:T ratio specified ($P < .05$).

As shown in figure 2, infusion of CS at a rate of 0.09 mg/kg/day did not elevate plasma CS levels above the preinfusion base-line level. Infusion of CS at a rate of 0.9 mg/kg/day led to a plasma CS level of approximately 60 ng/ml which was maintained over the duration of sampling. Infusion of CS at a rate of 9.0 mg/kg/day significantly elevated plasma CS levels to ~490 ng/ml on day 1 after beginning CS infusion. However, at this infusion rate, the level of CS declined on each successive day of sampling thereafter. By day 12 after the start of infusion, plasma CS had declined to ~50 ng/ml, a level that was equivalent to that observed in non-ADX mice sampled in the morning (Shimizu *et al.*, 1983; DeKrey *et al.*, 1993). These results indicated that elevation of plasma CS to ~500 ng/ml could be achieved in ADX mice with a CS dose of 9 mg/kg/day (and probably higher doses), but elevated steady state levels could not be maintained, and significantly elevated CS levels could not be maintained for longer than 3 to 4 days.

Study 3: The effects of CS infusion during three-day windows after alloantigen challenge. The intent of this study was to examine the time-dependent effects of HxCB-like plasma CS elevation on the generation of alloantigen-specific CTL activity *in vivo*. Osmotic pumps designed to infuse CS at a rate of 0 or 10 mg/kg/day for 3-day periods were implanted into mice on days 3 to 6 post alloantigen challenge. In this way, mice were exposed to CS on days 3 to 6, 4 to 7, 5 to 8 or 6 to 9. This schedule was based on the fact that HxCB did not induce elevated CS levels prior to day three (fig. 3).

As summarized in table 2, infusion of CS over days 3 to 9 had no significant effect on the generation of CTL activity measured either as % CTX or as LU/spleen. In contrast, spleen weights were significantly decreased in all CS-treated animals, and thymus weights were reduced in all CS-treated animals except those that were implanted with pumps on day 6 (table 2). Spleen cellularity was not significantly affected by CS exposure.

Study 4: Effects of CS or dexamethasone infusion on days 0 to 3 post alloantigen challenge. Inasmuch as no effective CTL suppression was observed in mice treated with CS after day 3 post alloantigen challenge, in this study we examined the effects of CS treatment before day 3. A dose rate of CS at 16 mg/kg/day was sufficient to cause suppres-

TABLE 1

Dose-dependent effects of CS infused for 14 days on LU/spleen, organ weights and plasma CS levels of alloantigen-challenged mice[a]

CS (mg/kg/day)	LU/Spleen	Body Weight[b] (BW) (g)	Spleen Weight[c] (mg/g BW)	Spleen Cells ($\times 10^7$)	Thymus Weight (mg/g BW)	Plasma CS (ng/ml)
0	481 ± 79	26.9 ± 0.6	6.0 ± 0.2	11.1 ± 0.7	0.91 ± 0.08	32 ± 2
1	414 ± 77	28.7 ± 0.5	5.7 ± 0.4	10.8 ± 0.5	0.79 ± 0.16	29 ± 3
2	547 ± 110	28.6 ± 0.7	5.6 ± 0.3	10.8 ± 0.6	0.53 ± 0.06[d]	52 ± 4
4	432 ± 138	25.2 ± 0.6	4.4 ± 0.2[d]	7.6 ± 0.5[d]	0.31 ± 0.03[d]	90 ± 10[d]
8	283 ± 127	25.7 ± 1.0	4.2 ± 0.5[d]	6.9 ± 0.7[d]	0.36 ± 0.13[d]	47 ± 8
16	175 ± 30	27.7 ± 0.3	4.6 ± 0.4[d]	8.0 ± 1.0[d]	0.39 ± 0.07[d]	42 ± 8

[a] Osmotic pumps were implanted into all CS-treated mice 4 days before alloantigen challenge as described in "Methods." All animals were killed on day 10 post alloantigen challenge. Values are given as mean ± SEM for six to eight animals per group.
[b] Indicates body weight on the day of necropsy. No significant difference in body weight change was observed over the course of the study.
[c] Organ weights are given as ratios of body weight.
[d] Indicates the mean is significantly different from untreated control by analysis of variance and post hoc Bonferroni *t* tests ($P < .05$).

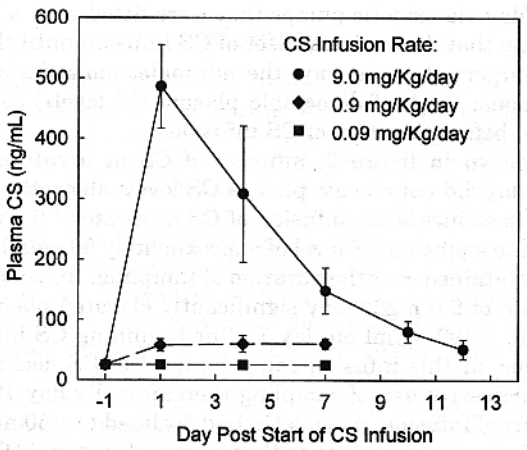

Fig. 2. Lack of prolonged plasma CS elevation after infusion of exogenous CS. Animals were adrenalectomized and implanted with osmotic pumps containing CS as described in "Methods." The pumps were designed to release CS at a constant rate for 14 days. The pumps were fitted with a length of tubing that delayed infusion of CS until 1 day after implantation. Serial blood samples were collected from the tails of mice on various days after ADX, and plasma was analyzed for CS levels by RIA. Data are presented as mean ± SEM for five to six mice per group.

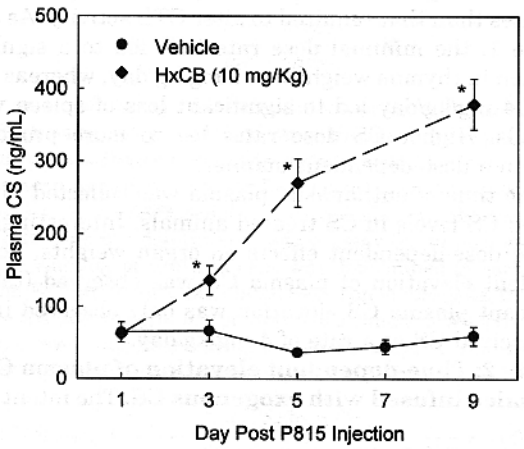

Fig. 3. HxCB treatment causes elevation of plasma CS levels beginning on day 3 post alloantigen challenge. Plasma CS levels in alloantigen-challenged mice are shown as adapted from DeKrey *et al.*, 1993. Data represent mean ± SEM for 6 to 10 animals per group. Mice were treated with HxCB at 0 or 10 mg/kg 1 day before i.p. injection with 1×10^7 allogeneic P815 cells. Animals were killed on the days indicated. For statistical purposes, plasma samples with CS levels below the lower limit of detection (25 ng/ml) were given the value 25 ng/ml. *Indicates a significant difference between treatment groups on the day specified ($P < .05$).

sion of CTL activity in study 1. Therefore, the same dose rate was used in this study. The effects of a high dose rate of dexamethasone (9.4 mg/kg/day), a known immunosuppressive GC (Exon *et al.*, 1990), were examined in a separate trial. Osmotic pumps designed to infuse vehicle, CS or dexamethasone for 3-day periods were implanted into mice approximately 6 hr before alloantigen challenge. CTL activity was measured 10 days later.

CS infusion at 16 mg/kg/day on days 0 to 3 had no suppressive effect on the generation of CTL activity measured either as % CTX (fig. 4A) or as LU/spleen (table 3). In contrast, dexamethasone treatment (9.4 mg/kg/day) over the same time period markedly suppressed CTL activity measured as either % CTX (fig. 4B) or LU/spleen (table 3). Interestingly, both CS and dexamethasone caused significant reduction of thymus weight and total spleen cells, with the effects of dexamethasone being more pronounced (table 3). CS, but not dexamethasone, caused significant reduction of spleen weight (table 3).

Study 5: Effects of CS on in vitro generated CTL. MLTC cultures were used to examine the direct effects of CS on the generation of CTL activity *in vitro*. As shown in figure 5A, addition of CS to MLTC cultures had a concentration-dependent suppressive effect on viable cell recovery. CS concentrations less than 10^{-8} M did not alter cell viability. However, the viability of cultured cells decreased markedly over the concentration range 10^{-8}-10^{-7} M CS, with cell recovery decreasing to <10% at higher concentrations. Viable cell recovery from cultures with 2.5×10^{-8} M and 5.0×10^{-8} M CS were 40% and 24% of vehicle-treated cultures, respectively.

Due to poor cell recovery, CTL activity could not be assessed in cultures with CS concentrations > 10^{-8} M. Therefore, the effects of CS between 10^{-8} and 10^{-7} M on the generation of CTL activity were examined. As shown in figure 5B, CTL activity (% CTX) was significantly suppressed in a concentration-dependent manner by exposure to CS. CS at a concentration of 2.5×10^{-8} M suppressed CTL activity by 74% (calculated as LU/culture). Because higher CS concentrations caused significantly lower cell recovery, CS at 2.5×10^{-8} M was used in subsequent experiments.

The time-dependent sensitivity of *in vitro*-generated CTL to CS exposure was examined by addition of CS to cultures on various days post culture initiation. As shown in table 4, *in vitro* generation of CTL activity was sensitive to CS exposure at 2.5×10^{-8} M when present from the day of culture initiation. In contrast, addition of CS on any later day did not

TABLE 2

Organ weights, CTL activity and LU/spleen of mice given CS during three-day windows after alloantigen challenge[a]

CS Infusion Period (Days)	CS (mg/kg/day)	Body Weight[b] (BW) (g)	Thymus Weight[c] (mg/g BW)	Spleen Weight (mg/g BW)	Spleen Cells ($\times 10^7$)	% CTX[d] (33:1)	LU/Spleen
3–6	0	23.1 ± 0.5	0.42 ± 0.03	5.5 ± 0.2	10.4 ± 0.7	54.7 ± 2.2	925 ± 60
	10	22.5 ± 0.3	0.27 ± 0.02[e]	4.7 ± 0.3[e]	9.4 ± 0.5	55.9 ± 1.3	856 ± 79
4–7	0	23.5 ± 0.6	0.54 ± 0.07	4.9 ± 0.5	9.2 ± 0.7	48.4 ± 1.7	621 ± 62
	10	23.9 ± 0.2	0.25 ± 0.01[e]	3.9 ± 0.2[e]	8.8 ± 0.4	55.2 ± 2.9	773 ± 118
5–8	0	23.2 ± 0.4	0.44 ± 0.05	5.1 ± 0.2	9.4 ± 0.3	52.3 ± 1.1	705 ± 54
	10	22.3 ± 0.5	0.25 ± 0.02[e]	4.1 ± 0.2[e]	8.5 ± 0.4	51.0 ± 1.1	656 ± 63
6–9	0	22.8 ± 0.7	0.39 ± 0.04	5.6 ± 0.2	9.1 ± 0.8	48.4 ± 5.8	790 ± 83
	10	22.0 ± 0.6	0.31 ± 0.04	4.3 ± 0.2[e]	8.0 ± 0.4	53.7 ± 2.2	756 ± 100

[a] Osmotic pumps were implanted on the day indicated relative to alloantigen challenge as described in "Methods." All animals were killed on day 10 post alloantigen challenge. Values indicate mean ± SEM for five to seven animals per group.
[b] Indicates the body weight on the day of necropsy. No significant difference in body weight change was observed between treatment groups over the course of the study.
[c] Organ weights are given as ratios of body weight.
[d] Indicates % CTX at an E:T ratio of 33:1.
[e] Indicates the mean is significantly different from vehicle-treated control ($P < .06$).

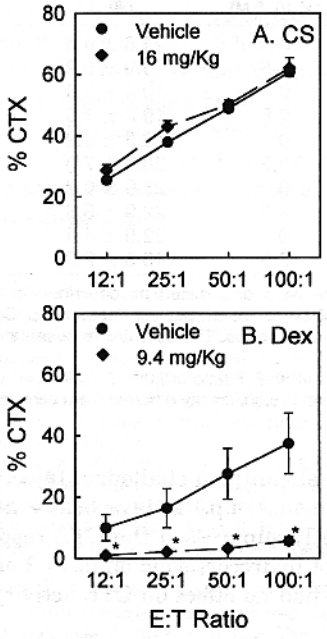

Fig. 4. Effects of CS or dexamethasone (Dex) infusion on days 0 to 3 post alloantigen challenge. CTL activity is presented as % CTX at various E:T ratios (mean ± SEM) for 5 to 7 mice per group. Animals were implanted with osmotic pumps containing CS, Dex or vehicle 6 hr before alloantigen challenge. The pumps were designed to release CS at a constant rate for 3 days. Animals were killed on day 10 post alloantigen challenge and splenic CTL activity was measured as described in "Methods." *Indicates the mean is significantly different from vehicle-treated control at the E:T ratio specified ($P < .05$).

alter CTL activity (table 4). The level of CTL activity generated in vehicle-treated cultures was consistent across days of vehicle addition and was the same as in nonvehicle-treated cultures (data not shown).

Discussion

Elevation of endogenous CS has been linked to suppression of immune function after stress or exposure to xenobiotic compounds (Pruett *et al.*, 1993). However, no previous studies have directly examined the immunological effects of CS elevation to the approximate levels observed in stressed or xenobiotic-exposed mice. The intent of the studies reported here was 1) to determine if CS infusion could significantly elevate plasma CS to levels induced by stress or xenobiotic exposure and 2) to examine the effects of such CS elevation on CTL activity.

Infusion of CS at a dose rate of 9 mg/kg/day was sufficient to elevate plasma CS levels to ~500 ng/ml on the day after the start of CS infusion. However, plasma CS levels steadily declined over the following 11 days to a level of ~50 ng/ml. Morning plasma CS levels of ~50 ng/ml are normal for non-ADX mice (Shimizu *et al.*, 1983; DeKrey *et al.*, 1993). Although the mechanism underlying the decrease in plasma CS levels in ADX mice is not known, induction of drug metabolism is a likely cause. Induction of drug metabolizing enzymes is a classic response after treatment with many drugs including glucocorticoids (Gibson and Skett, 1986; Juchau, 1990). Although the results obtained using ADX mice cannot be directly compared with those from intact (non-ADX) mice, the fact that low (~60 ng/ml) plasma CS levels were maintained in ADX mice infused with CS at 0.9 mg/kg/day suggests that high dose CS infusion may be required for elevation of CS clearance rates. Interestingly, the highest plasma CS level observed in intact mice after 14 days of CS infusion occurred at an infusion rate of 4 mg/kg/day. These results suggest that a CS infusion rate >4 mg/kg/day may be required for elevation of CS clearance rates.

Because plasma CS levels are not significantly elevated in HxCB-treated mice until 3 days after alloantigen challenge, we first examined the direct effects of exogenous CS treatment on CTL activity by beginning CS infusion 3 days after alloantigen challenge. Based on the results of the studies discussed above, infusion of CS at 10 mg/kg/day was expected to elevate plasma CS to peak levels of ~500 ng/ml, similar to the peak CS levels observed in HxCB-treated mice [200 to 550 ng/ml (DeKrey *et al.*, 1993; Kerkvliet *et al.*, 1990)]. However, because significantly elevated plasma CS levels could not be expected for much longer than 3 days, we examined the effects of CS infusion during 3-day windows of time. Interestingly, at all times CS infusion had no effect on CTL activity. These results support the conclusion that CS elevation in HxCB-treated animals does not mediate the suppression of CTL activity (DeKrey *et al.*, 1993).

The only significant, albeit slight, effect of CS on CTL activity in the *in vivo* studies described here was observed when CS infusion was begun 4 days before alloantigen challenge (16 mg CS/kg/day for 14 days). Due to the time-depen-

TABLE 3

LU/spleen and organ weights of mice treated with CS or dexamethasone (Dex) on days 0–3 post alloantigen challenge[a]

Day of Pump Implantation	Glucocorticoid (mg/kg/day)	LU/Spleen	Body Weight[b] (BW) (g)	Spleen Weight[c] (mg/g BW)	Spleen Cells ($\times 10^7$)	Thymus Weight (mg/g BW)
0	0	892 ± 109	23.9 ± 0.6	5.8 ± 0.3	14.0 ± 0.9	0.58 ± 0.07
	16 (CS)	726 ± 110	23.1 ± 0.7	4.2 ± 0.2[e]	9.4 ± 0.8[e]	0.37 ± 0.04[e]
0	0	278 ± 157	23.9 ± 0.6	4.4 ± 0.6	9.8 ± 1.4	1.46 ± 0.21
	9.4 (Dex)[d]	1.3 ± 0.3[e]	25.9 ± 0.4[e]	3.2 ± 0.4	2.1 ± 0.6[e]	0.22 ± 0.02[e]

[a] Osmotic pumps were implanted approximately 6 hr before alloantigen challenge as described in "Methods." All animals were killed on day 10 after alloantigen challenge. Values indicate mean ± SEM for five to seven animals per group.
[b] Indicates the body weight on the day of necropsy. A significant difference in body weight was reflective of body weight change over the course of the study.
[c] Organ weights are given as ratios of body weight.
[d] The total dose of dexamethasone is indicated. Animals that received dexamethasone were implanted with two osmotic pumps each.
[e] Indicates the mean is significantly different from vehicle-treated control ($P < .05$).

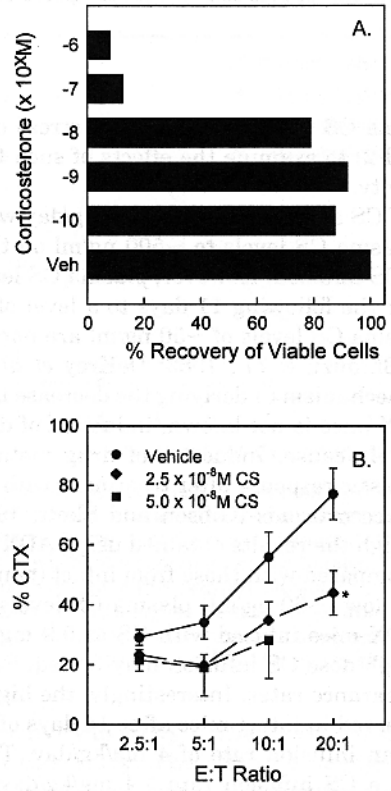

Fig. 5. CS exposure in vitro is cytolytic and immunosuppressive. Spleen cells (1.5×10^7) were cultured with 3×10^5 mitomicin C-treated P815 cells in the presence of CS from the time of culture initiation. After 5 days, the CTL activity of each culture was determined with E:T ratios based on viable cell numbers. The viability of cells (A) is given as percent of control (0.02% ethanol) for duplicate cultures. The CTL activity (% CTX) against ^{51}Cr-labels P815 cells (B) is given as mean ± SEM of six replicate cultures. CTL activity at an E:T ratio of 20:1 could not be determined for cultures containing 5.0×10^{-8} M CS due to low cell viability (B). *Indicates the mean is significantly different from vehicle-treated control at the E:T ratio specified ($P < .05$). LU/culture for vehicle and CS (2.5×10^{-8} M) exposed cultures were 76.7 ± 15.5 and 19.9 ± 5.0 [mean ± SEM], respectively, and were significantly different ($P < .05$).

dent decrease of plasma CS levels (fig. 2), it is unlikely that significant plasma CS elevation persisted for more than 5 days from the time of pump implantation. However, because infusion of CS at the same dose rate on days 0 to 3 relative to alloantigen challenge had no effect on CTL activity, it suggests that the days before antigen challenge may be the most sensitive for CS-induced CTL suppression. This conclusion is supported by the findings of Conlon *et al.* (1985) who reported that the sensitivity of mice to GC-induced CTL suppression

TABLE 4

Time-dependent effects of CS on CTL activity and LU/MLTC culture[a]

Day of CS Addition	CS ($\times 10^{-8}$ M)	% CTX[b] (20:1)	LU/Culture
0	0	21.6 ± 4.5	11.2 ± 2.9
	2.5	9.9 ± 0.2[c]	1.7 ± 0.8[c]
1	0	19.4 ± 3.9	11.5 ± 4.0
	2.5	19.2 ± 7.9	6.7 ± 3.7
2	0	20.5 ± 2.9	12.6 ± 2.4
	2.5	28.4 ± 7.9	13.8 ± 5.6
3	0	22.9 ± 0.9	13.2 ± 2.3
	2.5	22.9 ± 5.8	11.2 ± 4.3
4	0	22.5 ± 4.4	13.0 ± 3.6
	2.5	19.8 ± 2.2	7.2 ± 1.4

[a] LU/MLTC culture were determined as described in "Methods" for three cultures (separate spleen cell pools) per treatment group. Spleen cell pools were shared across treatment groups. The data are representative of three separate experiments.
[b] Indicates % CTX at an E:T ratio of 20:1.
[c] Indicates the mean is significantly different from vehicle-treated control ($P < .05$).

decreased after alloantigen challenge. Injection of hydrocortisone (2.5 mg/mouse, i.p.) 2 days before alloantigen challenge significantly suppressed the CTL response. However, the same dose of hydrocortisone on days 2 or 7 after alloantigen challenge had no effect on CTL activity (Conlon *et al.*, 1985).

In contrast to infusion of CS, infusion of dexamethasone at 9.4 mg/kg/day caused a marked suppression of CTL activity when mice were exposed on days 0 to 3 relative to alloantigen challenge. These data indicate that the CTL response is not insensitive to GC-induced suppression during that time. Indeed, the CTL response to alloantigen in mice has been shown to be sensitive to dexamethasone-induced suppression as late as 9 to 11 days after antigen challenge (Borel, 1976). Dexamethasone is >50-fold more potent as an immunosuppressive GC than corticosterone (Schleimer *et al.*, 1984). Therefore, these data suggest that higher doses of CS than used in our study, if given after alloantigen challenge, might also suppress CTL activity.

The generation of CTL *in vitro* was suppressed in the presence of CS. When added at the initiation of MLTC cultures, 2.5×10^{-8} M CS was sufficient to suppress CTL activity by 43%. However, when CS was added to MLTC cultures on any subsequent day, no suppressive effect on CTL activity was observed. Similar results were reported by Suehiro (1987) who showed that CTL activity in mixed lymphocyte cultures could be suppressed by dexamethasone (1×10^{-8} M) if added on the first day of culture, but no suppression of CTL activity was observed when dexamethasone was

added 48 hr later at a 100-fold higher concentration. Taken together, these results suggest that the CTL response is most sensitive to GC-induced suppression when exposure occurs near to the time of antigen challenge.

In their review of stress responses and immune suppression, Pruett *et al.* (1993) suggested that "suppression of one or more immunological parameters is reasonably consistent when peak CS levels exceed ~200 ng/ml and when assessment of immunological parameters is done at least 6–12 hours after exposure of the animals to the stressor." In the studies described here, there is reasonable confidence that the dose rate of CS administered to mice was sufficient to elevate plasma CS to stress-like levels, well in excess of 200 ng/ml, in all *in vivo* experiments. However, only a slight *in vivo* effect was observed. These results suggest that the alloantigen-specific CTL response may be less sensitive to plasma CS elevation than are other immune responses. However, CS is not the only mediator of stress-induced immune suppression (Pruett *et al.*, 1993). Therefore, an alternative interpretation of these results is that CS infusion (*i.e.*, separate from a stress response) may be less potent as an immunosuppressive agent *in vivo* than when combined with other mediators of the stress response. Further study will be required to understand the effects of interacting stress hormones on immune function.

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