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REDUCTION IN TUMOR NECROSIS FACTOR RECEPTOR AFFINITY AND CYTOTOXICITY BY GLUCOCORTICOIDS

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Summary: Micromolar concentrations of glucocorticoids rendered L-M cells (a murine tumorigenic fibroblast line) less sensitive to the cytotoxic activity of murine TNF. The potency of different steroids paralleled their known anti-inflammatory potency, and pretreatment was more effective than post treatment. Sex steroids and mineralocorticoids were ineffective. Dexamethasone also decreased the sensitivity of MCF-7 (a human mammary carcinoma line) to the cytotoxic activity of human recombinant TNF. Pretreatment of both cell lines reduced the affinity of specific cell surface receptors for the binding of their species ^{125}I-TNF about 3-fold while retaining the same number of binding sites. The decrease in sensitivity was not due solely to the inhibition of early TNF-induced events (such as binding, internalization or signal transduction). Dexamethasone modestly enhanced inhibition beyond that of neutralizing antiserum alone when both were added midway in the L-M killing reaction (after receptor down regulation but before the onset of complete cell death).

Corticosteroids have a broad range of immunomodulatory properties in vivo and in vitro in man (1). Corticosteroids repress the production of tumor necrosis factor/cachectin (TNF) in response to lipopolysaccharide in vivo (2) and in vitro (3). (TNF is an endogenous mediator of septic shock (4)). Corticosteroids may be efficacious when given early to patients undergoing septic shock (5). The concentrations that are required to repress TNF synthesis in vitro are low relative to the massive amounts that are given in shock therapy. This discrepency implies that high-dose therapy may induce some of its benefits by means other than the inhibition of the production of leucocyte mediators.

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Dexamethasone (6) and hydrocortisone (7) have recently been observed to inhibit TNF-mediated cytotoxicity in vitro. These studies implicated phospholipase A2 in the mechanism of cytotoxicity. Since cytotoxicity measurements are subject to caveats (8) and the mechanism of glucocorticoids is complex, detailed in vitro studies were undertaken to examine the effects of steroid treatment on cell lines known to be sensitive to the cytotoxic activity of TNF. Micromolar concentrations of glucocorticoids were found to reduce the affinity of the receptor and to render sensitive cells nearly refractory to the cytotoxic activity of TNF. The results have implications for other TNF-mediated activities.

MATERIALS AND METHODS

Murine TNF (mTNF), also called necrosin, was isolated from J774.1 cell line supernatants as described (9). Human recombinant TNF (hTNF) was a generous gift of the Cetus Corp. Standard hTNF (lot 86/659) and recombinant human lymphotoxin were obtained from the Natinonal Institute for Biological Standards and Control, Hertfordshire, UK. Relative to the standards, the TNF preparations used herein had the following specific activities as measured on L-M cells in the presence of 4 uM cycloheximide (8): 8×10^5 U/ug, mTNF; 3×10^4 U/ug, hTNF. Preparations were radioiodinated to specific activities of 0.3-0.5 mCi/nmole. Iodinations and equilibrium specific binding were performed as described previously (10). Anti-mTNF antiserum was generated in New Zealand white rabbits by conventional methods. The antisera were judged monospecific with respect to other J774.1 products as assessed by $NaDodSO_4$ electrophoresis of crude supernatants followed by immunoblotting and also by specific immunoprecipitation from crude supernatants. Anti-mTNF neutralized mTNF and hTNF but not lymphotoxin. The TNF

L-M (CCL 1.2), a murine tumorigenic fibroblast, and MCF-7 (HTB-22), a human mammary carcinoma, were obtained from the American Type Culture Collection. They were cultured at 38° in a humidified atmosphere of 95% air/5% CO2 in Eagle's minimum essential medium with Earle's salts that contained 5% (iron) supplemented calf serum (Hyclone). MCF-7 medium lacked phenol red and was additionally supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.1 mM glutamine, and 10^{-9} M estradiol. The sensitivity of these lines to TNF has been studied previously (8-11). Survival and ^{51}Cr release titration curves were performed similarly to those previously described (8). Steroids were obtained from Sigma. They were dissolved in dimethylsulfoxide, diluted in growth medium and added to cultures such that the final concentration of dimethylsulfoximide (diluent) was less than 0.1%.

RESULTS AND DISCUSSION

L-M and MCF-7 cells were killed during prolonged exposures to TNF. Murine TNF or hTNF was titered on growing L-M or MCF-7 cells respectively simultaneously with the addition of varying concentrations of dexametha-

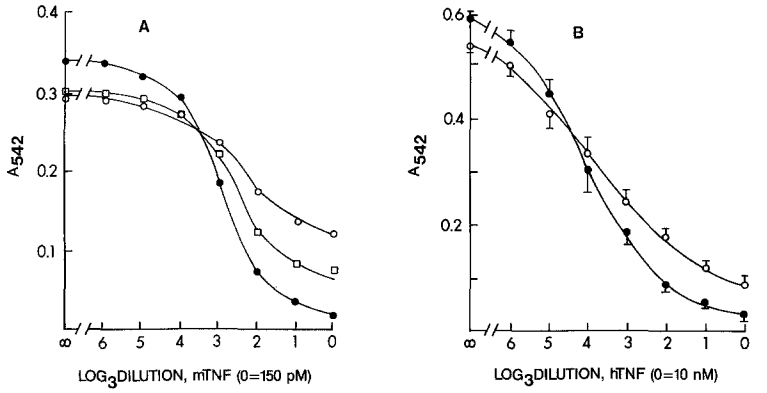

Fig. 1. The effect of dexamethasone on TNF survival. The indicated species TNF was added with or without dexamethasone (●, diluent; o, 10 uM; □, 1 uM) to growing cells: A. 3×10^4 L-M, B. 5×10^3 MCF-7 in a total volume of 250 ul medium/0.3 cm^2 well. The cultures were incubated at 38° in a humidified atmosphere of 95% air/5% CO_2 for A. 20 hrs or B. 48 hrs. They were treated with 0.006% neutral red for 1-2 hrs., washed with physiologic saline, lysed, and A_{542} determined. L-M points are the mean of duplicate wells. MCF-7 points are the mean of quadruplicate wells. Bars indicate the SEM. Figures are representative of replicate trials.

sone (Fig. 1). Two effects were observed. Firstly, dexamethasone enhanced survival (inhibited cytotoxicity) as indicated by the deflection in the titration curve at high concentrations of TNF. The inhibition of cytotoxicity was confirmed by light microscopic examination and also by a decreased release of ^{51}Cr (described in greated detail below). Ten uM dexamethasone was substantially refractory in L-M cells, and it was more refractory than 1 uM. Secondly, the treatment was modestly growth inhibitory as reflected by the slight decrease in A_{542} in the absence of TNF. (Growth repression of L929 cells, the parent of L-M, has been noted previously (12)). Both dexamethasone effects were apparent but less dramatic in MCF-7 cells. The relative responsiveness of the cell lines was in accord with the sensitivity of their species of origin (murine > human) to glucocorticoids in general (1).

Both of the dexamethasone effects were time and concentration dependent. Pretreatment was more inhibitory than posttreatment. When L-M cells were pretreated with dexamethasone for 24 hrs, the mTNF titration curves tended to flatten (Fig. 2). Simultaneous treatment with both dexamethasone and 4 uM cycloheximide produced titration curves that were

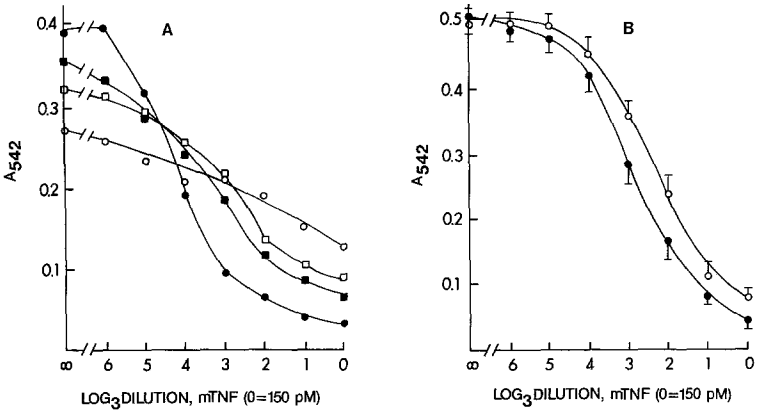

Fig. 2. The effect of time of treatment on survival. Survival titrations were carried out on L-M cells as described in the legend to Fig. 1, except the indicated concentrations of dexamethasone were added A. 24 hrs prior to or B. 6 hrs after mTNF. Post determinations were carried out in quadruplicate. Bars indicate the SEM. Figures are representative of duplicate trials. ●, diluent; ■, 0.1 uM; □, 1 uM; o, 10 uM.

identical to cycloheximide alone (results not shown). Thus, dexamethasone was not refractory in the presence of cycloheximide, and its protective effect may have required transcriptional or translational events.

^{51}Cr release from prelabeled L-M cells was used as a more direct indicator of cytotoxicity. Representative results are shown in Fig. 3.

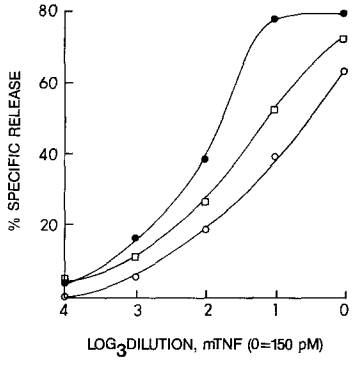

Fig. 3. The effect of glucocorticoids on ^{51}Cr release. L-M cells were prelabeled by overnight incubation with 1 uCi ^{51}Cr/ml/10^5cells/2 cm^2 well. The wells were washed 3 times and filled with medium containing the indicated concentration of mTNF with or without glucocorticoid (●, diluent; o,1 uM dexamethasone; □, 1 uM prednisolone). After 20 hrs, 0.5 ml supernatant was removed and its radioactivity determined. Points are the mean of duplicate wells and are expressed as percent maximum release using the following formula: ((treatment release - spontaneous release)/ (total release - spontaneous release)) X 100, where spontaneous release is the control (no mTNF) radioactivity, treatment refers to mTNF, and total release is that radioactivity recovered from cultures that were lysed with detergent.

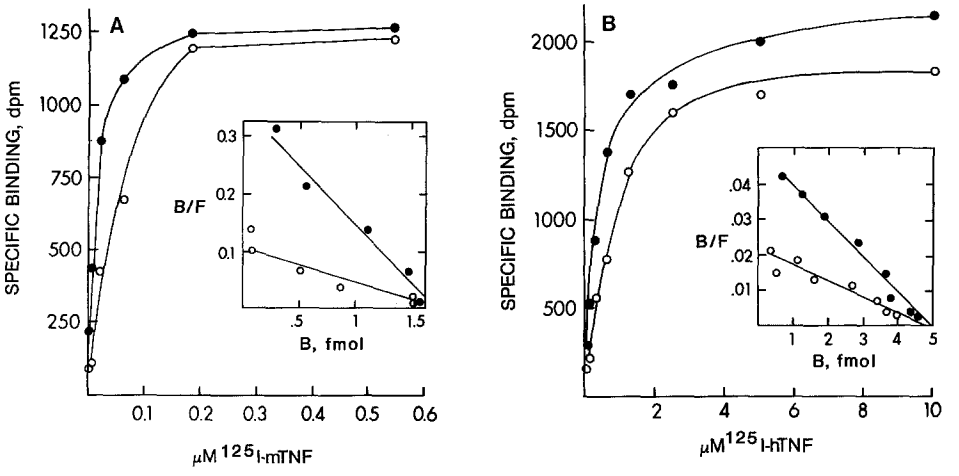

Fig. 4. Equilibrium specific binding. A. L-M (4 x 10^5/2 cm^2well) or B. MCF-7 (10^6/10 cm^2 well) were incubated with (o) or without (●) 10 uM dexamethasone for 12 hrs. Wells were washed 3 times with cold binding medium (MEM, 1% calf serum, 10 mM Hepes), and then binding was carried out by gently rocking culture trays with A. ^{125}I-mTNF or B. ^{125}I-hTNF respectively in a volume of 0.2 ml/cm^2 in the cold for 6 hrs. The trays were washed 3 times with cold binding medium, cells were solubilized with 1% detergent and radioactivity determined. Points show specific binding which was the average of duplicate wells subtracted from a companion well that contained a 200-fold excess of unlabeled ligand (nonspecific binding). Inset, Scatchard analysis.

Treatment with 1 uM dexamethasone was found to deflect the titration curve, and dexamethasone was superior to prednisolone. The degree of the deflection was dose dependent (results not shown). When other steroids were compared in this fashion, the results indicated that the capacity to deflect the titration curve was restricted to glucocorticoids. Their effectiveness was roughly proportional to their anti-inflammatory potency (13): dexamethasone, prednisolone, cortisol. Concentrations up to 10 uM of corticosterone and the sex steroids testosterone, estradiol and diethylstilbesterol were without effect in this analysis.

Dexamethasone pretreatment was found to reduce the affinity of cell surface receptors for ^{125}I-TNF. L-M and MCF-7 cells were pretreated with 10 uM for 12 hrs and then examined for the binding of ^{125}I-TNF (Fig. 4). Both lines demonstrated specific high affinity binding and saturation similar to previously published reports (10,11). The apparent K_ds were 10 pM for mTNF on L-M cells and 200 pM for hTNF on MCF-7. Dexamethasone reduced the appparent K_d approximately 3-fold without reducing

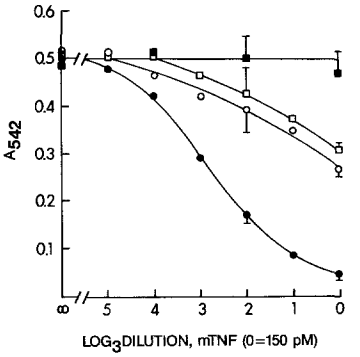

Fig. 5. Effect of belated dexamethasone and neutralizing antiserum on survival. TNF titrations were carried out on L-M cultures as described in the legend to Fig. 1, except that a 1:5000 final dilution of serum or 10 uM dexamethasone were included at 0 and 6 hrs into the cytotoxic reaction: ●, preimmune serum at 0 hrs and culture medium at 6 hrs; ■, antiserum at 0 hrs and culture medium at 6 hrs.; o, culture medium at 0 hrs and antiserum at 6 hrs.; □, culture medium at 0 hrs and dexamethasone and antiserum at 6 hrs. Points are the mean of triplicate wells and bars indicate representative SEMs.

the number of binding sites. The number of binding sites was determined from Scatchard analysis to be approximately 2000/cell for both cell lines.

Receptors were largely cleared from the surface of L-M cells 6 hrs after exposure to mTNF as cell death commenced (submitted). In order to gain some insight into whether glucocorticoids might affect events in the cytotoxic process that were more distal to binding, dexamethasone was added 6 hours into the course of the killing reaction together with neutralizing antiserum. Cell number was assessed after 20 hours (Fig. 5). By staining at 20 hours, the outcome of cytostatic and cytotoxic events that commenced 0 - 6 hours was revealed. Expectedly, neutralization was incomplete when the antiserum was added 6 hrs after TNF. The coaddition of dexamethasone shifted the titration curve slightly to the right, and while the shift was slight, it was reproducibly observed.

These studies confirm recent observations that glucocorticoids render cells less sensitive to TNF-induced cytotoxicity in vitro (6,7), which implicates phospholipase A2 in the mechanism of cytotoxicity (6,7,15). However, chlorpromazine and other inhibitors of phospholipase A2 did not inhibit the cytotoxicity of L-M cells (unpublished). Indomethacin and oxyphenbutazone (inhibitors of arachidonate metabolism) were also not

effective (8). Herein, the results suggest that the protective effect of glucocorticoids may be described as trophic. Pretreatment was more effective than post-treatment perhaps for two reasons: effectiveness may have required transcriptional or translational events, and effectiveness may have been mediated, at least in part, at the TNF receptor level. Glucocorticoids may reduce the TNF receptor affinity by altering its lipid milieu. Lipases and membrane composition may also contribute to distal aspects of cytotoxicity. The latter stage(s) were modestly inhibited.

A variety of in vitro activities that have been ascribed to TNF (14). All TNF responses probably begin with the binding of TNF to specific receptors. Chemical crosslinking experiments indicate that the receptors of different cell types have similar molecular characteristics (10,11, 16-19), and ligand affinity correlates with responsiveness (19-21). Dexamethasone was found to decrease the affinity of the cell surface receptors of two different cell lines for ^{125}I-TNF. Since binding to receptors may be common to the pleiotypic responses of TNF, glucocorticoids may affect other responses besides cytotoxicity. Thus, diminished in vivo cellular responses to leucocyte mediators may contribute to the mechansim of high-dose glucocorticoid therapy in septic shock.

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