THE EFFECTS OF IN VIVO HYDROCORTISONE ON LYMPHOCYTE-MEDIATED CYTOTOXICITY

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To examine the effects of in vivo hydrocortisone sodium succinate (HC) on natural killer (NK) cell and antibody-dependent cellular cytotoxicity (ADCC), 11 normal adults received a single intravenous bolus of 400 mg hydrocortisone. Lymphocytes were tested for NK activity and ADCC using ^{51}chromium (^{51}Cr)-release and single cell cytotoxicity assays against Molt-4 and sensitized RL ○→ target cells, respectively. Four hours after injection, both NK and ADCC activity were transiently increased in the ^{51}Cr-release system ($P < 0.05$). At 4 hours, there was a twofold increase in the relative frequency of potentially cytotoxic target binding cells ($P < 0.001$) but the absolute number of these cells did not change ($P < 0.1$). However, the percentage lysis of bound targets at 4 hours was not altered ($P > 0.1$). These data suggest that: 1) lymphocytes participating in NK and ADCC reactions are refractory to the kinetic and functional effects of HC; 2) the increased lytic activity observed at 4 hours is due to a selective depletion of noncytotoxic cells from the circulation; and 3) NK and ADCC activity did not differ in their responses to HC.

In recent years considerable effort has been directed to the study of the mechanisms and importance of the natural killer (NK) cell and antibody-dependent cellular cytotoxicity (ADCC) systems in health and disease (reviewed in ref. 1). Although a variety of defects of lymphocyte-mediated cytotoxicity have been described in autoimmune and malignant states, the influence of the pharmacologic agents used to treat these entities on this lytic activity has not been delineated.

Corticosteroids are one of the primary therapeutic modalities used in immunologically mediated disease and the effects of these agents on many lymphocyte-dependent processes have been described (2–4). However, these studies have not fully addressed the actions of corticosteroids on the circulatory kinetics and functional capabilities of cytotoxic lymphocytes. In part, these efforts have been hampered by both the lack of precise characteristics of lytic cells as well as the lack of methodologies permitting the exact determination of cytotoxic activity at the cellular level. The present study has used new techniques to circumvent these problems and we have now described the effects of in vivo hydrocortisone on the trafficking and intrinsic activity of cells participating in natural killer and ADCC reactions.

MATERIALS AND METHODS

Subjects. Eleven normal adults (5 females, 6 males; ages 20–45) participated in this study. The protocol for intravenous hydrocortisone administration has been reported elsewhere (2). Briefly, each volunteer received a single

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400 mg intravenous bolus of hydrocortisone sodium succinate (HC) (The Upjohn Co., Kalamazoo, MI; generous gift of Randall Crawson). Heparinized peripheral venous blood was drawn before (0 hour), and 4, 24, and 48 hours after hydrocortisone administration.

Cell suspensions. Purified mononuclear cells were prepared from heparinized blood samples by standard Ficoll-Hypaque density centrifugation and cell suspensions were depleted of adherent cells by passage over nylon wool columns (5). These cells were used as effector cells in all experiments.

Target cells. For the determination of natural killer activity, the human T cell line Molt-4 was employed. ADCC activity was quantitated with the murine T cell leukemia line RL ○→ which was sensitized for 30 minutes at 37°C with a 1:100 dilution of rabbit anti-mouse brain antibody (Litton Bionetics, Kensington, MD). This dilution of antisera produced optimal ADCC activity in both the ^{51}chromium (^{51}Cr)-release and single cell cytotoxicity assays.

^{51}Cr-release assay. A previously described ^{51}Cr-release microcytotoxicity assay (6) against Molt-4 and sensitized RL ○→ cells was used. Ten thousand ^{51}Cr-labeled target cells were mixed with varying numbers of effector cells in V-shaped microtiter wells (Flow Laboratories, McLean, VA) to give final effector:target ratios ranging from 50:1 to 5:1. Spontaneous release of ^{51}Cr by target cells was determined by placing labeled target cells in microtiter wells in the absence of effector cells. Cultures were incubated at 37°C in 5% CO_2 in air at 100% humidity for 3 hours. Plates were then centrifuged and one-half of the supernatant was removed and counted in an automatic Searle gamma counter. Percent cytotoxicity (or percent ^{51}Cr-release) was determined by the formula: supernatant counts per minute minus spontaneous release cpm divided by total target cell cpm minus spontaneous release cpm × 100. Spontaneous release was less than 10% for both target cell lines.

Single cell cytotoxicity assay. This assay was performed by a previously described modification (7) of the method of Ullberg and Jondal (8). Briefly, 2×10^5 effector cells and unlabeled target cells were mixed in a total volume of 0.2 ml RPMI 1640 with 15% fetal calf serum (FCS) in a 3-ml round-bottom tube. Tubes were centrifuged and incubated at 37°C for 10–20 minutes. The cell mixture was then carefully added to 0.5 ml of 0.5% agarose in RPMI 1640 with 10 m*M* HEPES and the agarose-cell mixture poured onto 60-mm plastic Petri dishes (Falcon Plastics, Oxnard, CA). The plate was incubated as above for 3 hours, the media then removed, and 0.1% trypan blue added for 10 minutes, followed by washing with cold phosphate buffered saline and fixation with 1% formaldehyde.

The percentage of target binding cells (TBC) was determined by counting the number of lymphocytes binding to target cells in 200–500 counted lymphocytes. The percentage of TBC with dead bound target cells was determined by counting the number of dead targets in 50 or more effector-target conjugates. Spontaneous (or "background") target cell death was determined by counting the percentage of dead targets in the absence of effector cells.

Analysis of data and statistical methods. Calculations of cytotoxic functions were performed as outlined above and as previously described (7–9). Data from the ^{51}Cr-release assay were utilized to determine the maximum cytotoxic potential (Vmax) of a given effector population. When the number of target cells is plotted against cytotoxic activity in the ^{51}Cr-release assay, the dose-response curve resembles Michaelis-Menten enzyme substrate kinetics and is expressed as:

$$V = \frac{Vmax \times T}{Km + T}$$

where T is the initial number of target cells, V the number of killed target cells, and Vmax the number of target cells killed when T approaches infinity; that is, when the system is saturated with target cells. Km, the Michaelis constant, is the number of target cells that produces one-half of Vmax. Vmax and Km can be calculated using the Lineweaver-Burk equation:

$$\frac{1}{V} = \frac{Km}{Vmax} \times \frac{1}{T} + \frac{1}{Vmax}$$

In this equation, there is a linear relationship between 1/V and 1/T and the reciprocal values of V and T can be plotted and regression analysis used to determine Vmax and Km from the reciprocals of the *y* and *x* intercepts, respectively.

The percentage of "active" lytic cells, that is, lymphocytes with bound and dead targets, in the effector population was determined by multiplying the percentage of TBC with the percentage TBC with dead targets. The maximum recycling capacity (MRC), which estimates the number of target cells killed by an active lytic cell during the 3-hour assay, was determined by dividing Vmax by the absolute number of active lytic cells.

Data are reported as the mean ± standard error of the mean and were compared using the 2-tailed Student's *t*-test.

RESULTS

The effects of in vivo hydrocortisone on lymphocyte-mediated cytotoxicity. Lymphocytes were isolated from blood samples drawn prior to (0 hour), and 4, 24, and 48 hours, after hydrocortisone administration and assayed for natural killer and ADCC activity in ^{51}Cr-release assays. As depicted in Figure 1, at a 50:1 effector:target ratio, there was a significant increase in NK ($P < 0.05$) and ADCC activity ($P < 0.05$) at 4 hours after hydrocortisone injection compared with 0 hour values. By 24 hours after injection, ^{51}Cr-release values returned to levels comparable with those obtained before treatment (*P* values not significant). Similarly, lymphocytes isolated 48 hours after hydrocortisone injection displayed lytic activity not significantly different from pre-hydrocortisone levels (*P* value not significant).

Although these studies indicated that in vivo hydrocortisone augmented NK and ADCC activity, the mechanisms of these increases were unclear. Con-

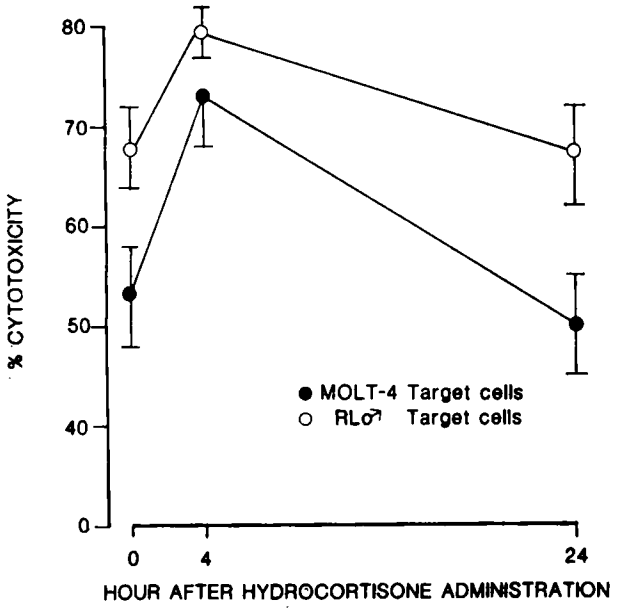

Figure 1. The effect of in vivo hydrocortisone on natural killer and antibody-dependent cellular cytotoxicity activity in the ^{51}Cr-release assay against Molt-4 and sensitized RL ♂→ target cells, respectively, at a 50:1 effector:target ratio. Data represent the mean ± SEM of 11 separate experiments. Similar results were apparent at all other effector:target ratios tested.

ceivably, lytic function could have been boosted by: 1) a relative or absolute increase in the number of cytotoxic effector cells, 2) an increase in the percentage of bound target cells lysed by a given effector cell, and/or 3) an increase in the number of target cells lysed by a given active effector cell during the assay period (i.e., the maximum recycling capacity).

The effect of in vivo hydrocortisone on cytotoxic effector cell circulatory kinetics. In this study, unlike previous ones, we were able to identify potentially cytotoxic effector cells by their ability to bind susceptible target cells. Lymphocytes isolated at each time point were assayed for binding capabilities with Molt-4 or RL ♂→ target cells. Before hydrocortisone administration, approximately 6–10% of nylon wool–purified lymphocytes were capable of binding Molt-4 or sensitized RL ♂→ target cells (Figure 2). Four hours after hydrocortisone administration at the time of relative lymphocytopenia ($P < 0.005$), there was a nearly twofold increase in the frequency of potentially cytotoxic lymphocytes capable of conjugating Molt-4 ($P < 0.001$) and RL ♂→ ($P < 0.001$) target cells. This effect was transient with a return to pretreatment levels of 24 hours (P values not significant versus 0 hour).

We then calculated the effects of in vivo hydrocortisone on the absolute number of target binding cells. As previously described (2,3), in vivo hydrocortisone induced a significant circulating absolute lymphocytopenia which was maximal at 4 hours after injection ($P < 0.001$ versus 0 hour values) (Figure 3). Again, as previously described (2,3), a rebound lymphocytosis was apparent at 24 hours ($P < 0.001$ versus 0 hour) with a return to pre-HC values by 48 hours (data not shown) (P value not significant).

When the absolute number of circulating target binding cells was calculated, the number of Molt-4 (P value not significant) and RL ♂→ (P value not significant) TBC was unchanged compared with 0 hour values. At 24 hours, there was a slight but insignificant increase in the number of these effector cells (P value not significant), reflective of the rebound total lymphocytosis observed. These values remained unchanged when reassessed at 48 hours.

The effect of in vivo hydrocortisone on the lysis of bound target cells. We next determined the effects of in vivo hydrocortisone on the lysis of target cells conju-

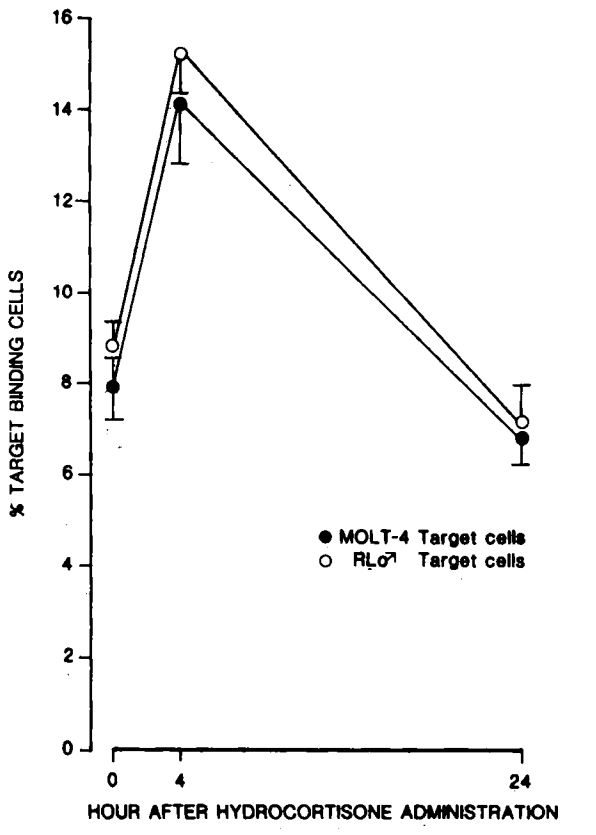

Figure 2. The effect of in vivo hydrocortisone on the relative frequency of Molt-4 and RL ♂→ target binding cells.

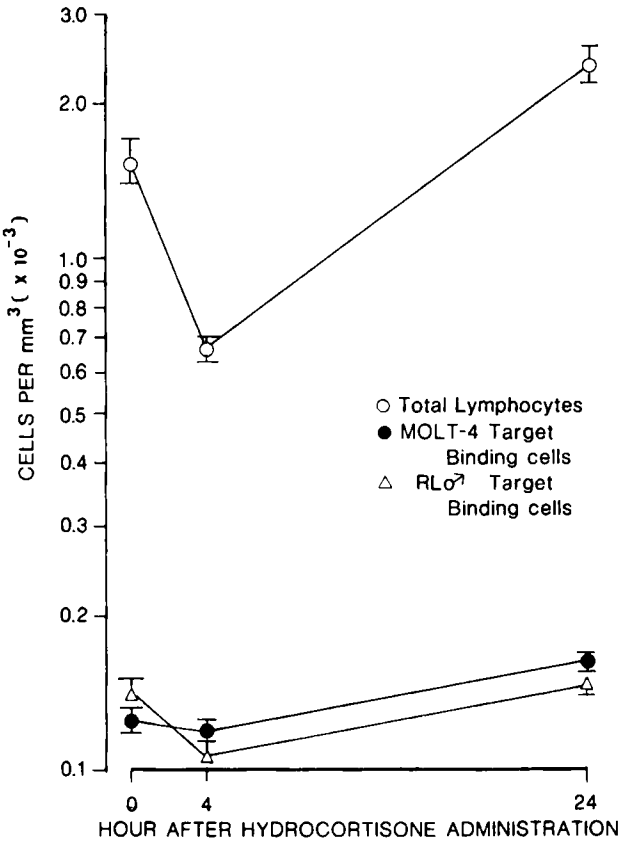

Figure 3. The effect of in vivo hydrocortisone on the absolute number of circulating lymphocytes, Molt-4 target binding cells and RL ♂ target binding cells.

gated to lymphocyte effector cells. Before injection, approximately 40% of Molt-4 and RL ♂ target cells bound to lymphocyte effector cells were killed as assayed in the single cell in agarose assay (Figure 4). Four hours after intravenous hydrocortisone, there was no alteration in the percentage of Molt-4 or RL ♂ TBC with dead bound target cells (P values not significant). Twenty-four hours after hydrocortisone injection, there was a slight but insignificant decrease in this parameter (P value not significant) and lytic values similar to baseline were observed at 48 hours (data not shown).

The effect of in vivo hydrocortisone on the maximum recycling capacity. The last cytotoxicity parameter to be determined was the maximum recycling capacity, which reflects the number of target cells destroyed by a given effector cell during the assay period (8). By combining data from the ^{51}Cr-release assay (i.e., Vmax) and the single cell cytotoxicity assay (i.e., percent active lytic cells), it is possible to estimate the maximum recycling capacity. As expected from ^{51}Cr-release data, the Vmax of natural killer and ADCC reactions was significantly increased at 4 hours. The Vmax of NK function rose from 9.4 ± 1.9 × 10^3 at 0 hour to 14.3 ± 1.9 × 10^3 at 4 hours ($P < 0.05$). Similarly, the pretreatment Vmax of ADCC activity increased from 8.4 ± 1.2 × 10^3 to 16.3 ± 1.4 × 10^3 at 4 hours ($P < 0.001$). Given the increased frequency of target binding cells at 4 hours, the percentage of active natural killer cells increased from 3.1 ± 0.3% at 0 hour to 5.8 ± 0.8% at 4 hours ($P < 0.001$). Likewise, the percentage of active ADCC effector cells was significantly increased from the pre-hydrocortisone levels of 3.4 ± 0.4% to the 4-hour level of 7.0 ± 1.3% ($P < 0.01$). These parameters were then utilized to calculate the MRC.

As depicted in Figure 5, in vivo hydrocortisone did not significantly alter the MRC of effector cells against either Molt-4 or RL ♂ target cells compared with pretreatment values (P values not significant versus 0 hour values).

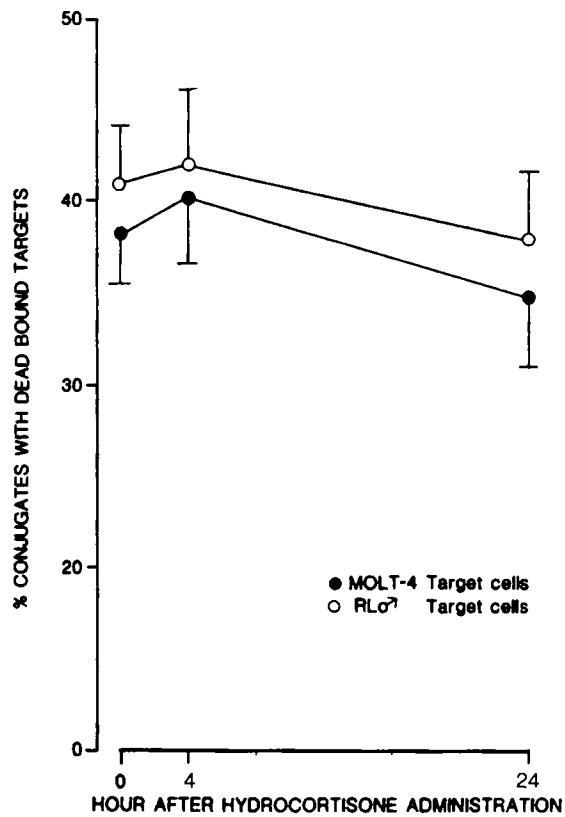

Figure 4. The effect of in vivo hydrocortisone on the lysis of bound target cells in the single cell cytotoxicity assay.

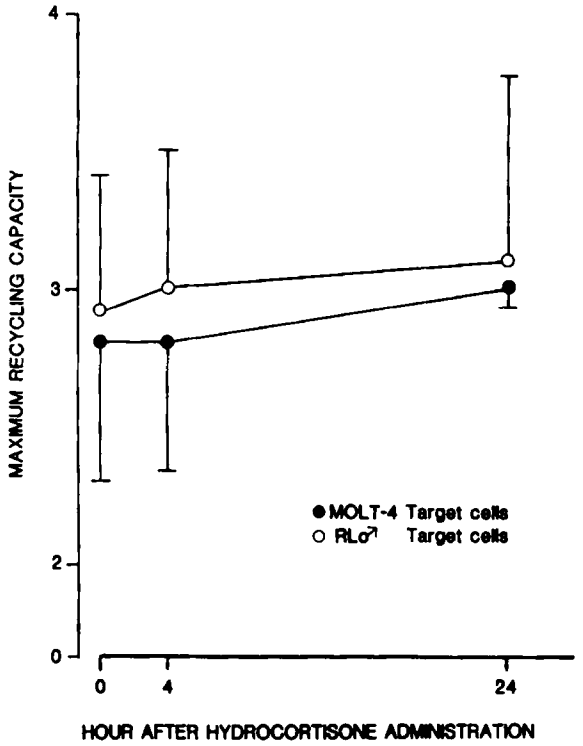

Figure 5. The effect of in vivo hydrocortisone on the maximum recycling capacity of active natural killer and antibody-dependent cellular cytotoxicity effector cells.

DISCUSSION

The present study has demonstrated the effects of hydrocortisone on the circulatory kinetics and lytic capabilities of cells participating in natural killer and ADCC reactions. Our investigations have indicated that intravenous hydrocortisone produces a significant but transient augmentation in lymphocyte-mediated cytotoxicity which occurs simultaneously with hydrocortisone-induced lymphocytopenia. By utilizing new single cell assay techniques to identify lytic cells by their ability to bind susceptible target cells, we have shown a doubling of the percentage of potentially cytotoxic target binding cells 4 hours after hydrocortisone administration. When the absolute numbers of circulating lymphocytes and TBC were compared, TBC were refractory to the depleting effects of hydrocortisone since these cells remained within the circulation while the majority of non-TBC migrated to extravascular locales.

When we examined NK and ADCC activity at the single cell level to determine if in vivo hydrocortisone altered the functional capabilities of these cells, no increase in the lysis of bound target cells was noted. Similarly, by using data from concurrently performed ^{51}Cr-release and single cell assays, we have shown that hydrocortisone does not affect the number of target cells killed by a given lytic lymphocyte during the assay period.

Of particular interest is our finding that natural killer and ADCC activity did not differ in their responses to hydrocortisone; however, other studies have shown somewhat different reactivity for natural killer and ADCC function. Although it has not been clearly determined that cells participating in these types of reactions are identical, our studies would indicate that they are at least similar in their hydrocortisone-induced circulatory kinetics and their functional unresponsiveness to this agent.

In studies not reported here, we have shown that the performance of cytotoxicity assays in the in vitro presence of physiologic ($10^{-6}M$), pharmacologic ($10^{-5}M$), or suprapharmacologic ($10^{-4}M$) concentrations of hydrocortisone (10) did not significantly affect effector cell viability, NK or ADCC activity (data not shown). These findings are compatible with our findings in that pharmacologic concentrations of hydrocortisone in vivo did not alter the intrinsic lytic capabilities of these lymphocytes. Furthermore, 18-hour preincubation of lymphocytes with these same concentrations of hydrocortisone failed to alter cytotoxic activity. These in vitro studies are at variance with those of Hoffman et al who demonstrated that 10^{-5} hydrocortisone could inhibit natural killer activity (11). However, the duration of lymphocyte preincubation with the drug and the effect on lymphocyte viability are not reported, and therefore we cannot reconcile the differences in our results.

Previous studies have reported a variety of effects of hydrocortisone on lymphocyte circulatory kinetics and functional activity (2–4,12). Corticosteroids induce a transient circulating lymphocytopenia with relatively greater effects on T cells compared with non-T cells (2–4,12). However, the precise effects of in vivo hydrocortisone on lytic lymphocytes that lack conventional T and B cell markers have been ill-defined. Furthermore, the usual nonspecific suppressive effects of hydrocortisone on many lymphocyte-dependent processes have not been adequately documented for cytotoxic activity.

Using conventional ^{51}Cr-release techniques, Parrillo and Fauci demonstrated that in vivo dexamethasone augmented ADCC at 4 hours, but produced no change in natural killer activity at this time (13). However, while ADCC values returned to baseline by

24 hours, NK activity was depressed at 24 and 48 hours with normalization by 96 hours. This study differs from ours in several respects. These authors used the long-acting preparation dexamethasone rather than the more conventionally utilized shorter duration corticosteroids such as hydrocortisone. Furthermore, Parrillo and Fauci used cell lines that have not generally been utilized in the study of cytotoxicity due to their relative resistance to lymphocyte-mediated lysis. Lastly, 18-hour incubation assays were employed. Since the majority of NK-derived lytic activity is completed by 3–4 hours, it is conceivable that effector cells other than those usually involved in natural killer and ADCC activity contributed to target cell lysis.

Onsrud and Thorsby assessed the effects of in vivo hydrocortisone on natural killer activity using a ^{51}Cr-release assay (14). These investigators showed increased natural killer activity at 4 hours after hydrocortisone but were unable to clearly identify the etiology of this augmentation. The increase in natural killer function did parallel an increase in the frequency of cells bearing Fc receptors for IgG but since other nonlytic mononuclear cells bear this receptor, the significance of this finding is unclear.

The use of the single cell assay system has also demonstrated that the increased 4-hour killer activity was not secondary to a hydrocortisone effect on the functional capabilities of lytic lymphocytes. From the in vivo data reported, it is evident that the intrinsic lytic machinery of cells mediating these reactions is resistant to corticosteroids. Although the precise mechanisms of this cytotoxic activity are unclear, a considerable body of evidence has accrued which favors lysosomal enzymes as the primary mediators of these reactions (reviewed in ref. 1). The reported effects of corticosteroids on the release of lysosomal enzymes from neutrophils have varied, making extrapolation of these data difficult. Persellin and Ku reported that these agents fail to stabilize lysosomes (15), a finding different from that of Wright and Malawista (16) and Ignarro (17), who demonstrated inhibition of enzyme release by corticosteroids in vitro.

In summary, our studies have shown that the lymphocytes mediating natural killer and ADCC reactions are resistant to the depletive and modulatory effects of hydrocortisone. Although increased lytic activity was apparent 4 hours after hydrocortisone administration, this augmentation was secondary to a relative increase in potentially cytotoxic target binding cells resulting from the exodus from the circulation of noncytotoxic cells. Analysis of killing at the single cell level failed to reveal any hydrocortisone-associated alteration in target cell lysis, indicating that the 4-hour increase in cytotoxicity could not be attributed to changes in effector function. Finally, we have demonstrated that the cells responsible for natural killer and ADCC reactions do not differ in their responses to hydrocortisone, a finding that strengthens the hypothesis that lysis of these 2 different types of targets is mediated by the same population of lymphocytes by perhaps similar corticosteroid-resistant mechanisms.

These studies, therefore, suggest that cells participating in natural killer and ADCC activity must now be considered kinetically and functionally corticosteroid-resistant. The etiology of the unresponsiveness of this unique subpopulation of lymphocytes and its possible implications in disease therapy must await further investigation.

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