Effect of Prednisolone on the Leukocyte Counts of Ponies and on the Reactivity of Lymphocytes In Vitro and In Vivo

S. P. TARGOWSKI

School of Veterinary Science and Medicine, Purdue University, Lafayette, Indiana 46207

Received for publication 26 June 1974

Treatment of ponies with a single dose of prednisolone markedly reduced the number of blood lymphocytes. A decrease of the number of eosinophils was also observed. In contrast, the number of neutrophils significantly increased. These profound changes were temporary and returned to the pretreatment level within 48 h. The number of monocytes did not show any of the significant changes post-prednisolone treatment. The reactivity of the blood lymphocytes of these ponies, in vitro, to stimulation with phytohemagglutinin (PHA) or streptokinase-streptodornase (SK-SD) was measured by incorporation of \(^{3}H\)thymidine by deoxyribonucleic acid of lymphocytes. The ponies' blood lymphocytes responded very well to PHA stimulation. The incorporation of \(^{3}H\)thymidine into deoxyribonucleic acid of the PHA-stimulated lymphocytes was 14 times greater than incorporation of the nonstimulated lymphocytes. SK-SD-stimulated lymphocytes incorporated only three times more \(^{3}H\)thymidine than nonstimulated lymphocytes. There was no significant difference in stimulation of lymphocytes with PHA or SK-SD before or after the prednisolone treatment. However, a significant decrease in the dermal response to the homologous antigen after this treatment in sensitive ponies was observed.

Corticosteroids are widely used in the treatment of a variety of diseases involving chronic inflammatory, neoplastic, or immunological processes. Despite this, little is known about the action of corticosteroids on the immune response in animals. Several immunosuppressive effects of these agents have been observed to occur in humans and animals, among these temporary lymphocytopenia (6, 8, 23), decreased immunoglobulin production (W. T. Butter and R. D. Rossen. Fed. Proc. 32:1028, 1973), impaired expression of cutaneous delayed hypersensitivity responses (5), activation of the latent infections (7, 17), and enhanced susceptibility of animals to infection (12, 13).

It is well established that the lymphocytes of laboratory animals and man consist of two major functional classes, the thymus-dependent T-cells and the thymus-independent B-cells. Each of these classes can probably be further divided into subpopulations, differing in particular immunological activity or stage of development. The majority of the peripheral T-cell population of lymphocytes are cortisone sensitive. A minor population of peripheral T-cells are relatively cortisone resistant. The population of B lymphocytes are apparently less cortisone sensitive than T-cells (8, 23).

The response of lymphocytes to different mitogens may serve as a marker for particular lymphocyte populations. Previous work has demonstrated that phytohemagglutinin (PHA) selectively activates T-cells and pokeweed mitogen activates T- and B-cells (38). In addition, concanavalin A stimulates column-adherent T-cells (4). Furthermore, there is some evidence that the subpopulation of the T-cells which responds to PHA is cortisone resistant, whereas 50% of the B-cell population (23) and the subpopulations of T-cells, such as concanavalin A-responsive lymphocytes, are cortisone sensitive (8).

In vivo administration of the corticosteroids markedly depletes concanavalin A- and pokeweed mitogen-responsive lymphocytes, whereas PHA-responsive lymphocytes remain unchanged in human blood (8). In the present study, the effect of in vivo cortisone administration upon cellular immune responses in ponies was investigated. During this investigation, the following parameters were measured: absolute number and types of circulating leukocytes, response of the lymphocytes to stimulation with mitogen or antigen, and dermal response to the injection of the streptokinase-streptodornase (SK-SD).

\(^{1}\) Present address: School of Health Related Professions, State University of New York at Buffalo, Buffalo, N. Y. 14214.
MATERIALS AND METHODS

Animals. Eight normal ponies and three ponies operated on for isolation of the intestinal loop a year ago were used in this investigation. The ponies were 2 years old and weighed from 100 to 125 kg. Five normal and three operated ponies received a single intravenous dose of 2 mg of azium (dexamethasone, a synthetic analogue of prednisolone; Schering Corp., Bloomfield, N.J.) per kg. Three normal ponies received a single intravenous infusion of 100 ml of saline. Venous blood samples were obtained 24 h prior to treatment, immediately before azium injection (zero time) and 2, 4, 7, 24, 48, 72, and 144 h after azium administration.

Total leukocytes and differential counts. Leukocyte counts were performed by using a Coulter counter (model FN, Coulter Electronics, Inc., Hialeah, Fla.). Differential counts were performed on a peripheral blood smear by the same observer throughout the study.

Separation of lymphocytes. Fifty milliliters of blood was drawn into heparinized syringes which were then supported in an upright position at ambient temperature for 20 min to allow the erythrocytes to sediment. The leukocyte-rich plasma was expressed into siliconized conical 35-ml tubes which contained an 8-ml Ficoll-Isopaque gradient. The gradient was prepared by dissolving 22.8 g of Ficoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden) in 240 ml of distilled water and adding 100 ml of Isopaque (sodium metrizate, 34%, Myegaard, Oslo, Norway). Each of the solutions was sterilized separately and mixed, and the pH was adjusted to 8 and kept at 4°C. The tubes with Ficoll-Isopaque gradient were overlaid by leukocyte-rich plasma and spun for 25 min at 800 × g at 20°C. The monolayer of cells at the plasma-Ficoll-Isopaque interface was removed with a Pasteur pipette and placed in a siliconized conical 10-ml tube. The cells were diluted with 5 ml of medium TC-medium 199 (Difco, Detroit, Mich.) and spun at 200 × g for 10 min at 4°C. The supernatant fluid was discarded and cells were washed twice more and then resuspended in 10 ml of medium. The number of cells was counted using the standard procedure. The standard preparation contained approximately 95% lymphocytes and 5% macrophages with 100% viability (trypan blue exclusion).

Preparation of cultures. The mononuclear cells obtained from the blood samples collected 24 h prior to treatment, immediately before azium injection (0 h), and 4, 7, 24, and 144 h after administration were washed three times in TC-medium 199. For culture, TC-medium 199 was supplemented with 0.02 M l-glutamine, 100 U of penicillin per ml (E. R. Squibb and Sons, Inc.), 100 U of streptomycin sulfate per ml (Pfizer Laboratories, New York), and 10% fetal bovine serum (Flow Laboratories, Rockville, Md.). Two sets of quadruplicate cultures were prepared from each of the blood samples. The concentration of cells was adjusted so that each culture contained 2 × 10^8 lymphocytes in a total volume of 2 ml of the medium. The mitogen was added to the one set of quadruplicate cultures in a previously determined optimal stimulatory dose, while another set served as a control. The optimal dose of the PHA lot 0528-56 control 585786 (Difco Laboratories, Detroit, Mich.) was 0.01 ml/culture. Leukocytes were obtained from the leukocyte-rich plasma by centrifugation, washing three times, and then distributing in two sets of quadruplicate cultures at a concentration of 10^6 cells/ml. SK-SD serial no. (1) 353-707 (American Cyanamid Co., Princeton, N.J.) was added in the optimal dose (streptokinase, 25 U, and streptodornase, 6.25 U) into each tube of one of the quadruplicate culture set. Another set served as a control. One microcurie of [3H]thymidine (Schwarz/Mann, Orangeburg, N.Y.) was added to each culture tube 18 h before harvesting. All the cultures were incubated 72 h in 5% CO_2 and then washed, and the incorporated isotope was counted (3).

Skin test. The injection sites were two shaven areas approximately 10 cm² arranged in a rough triangle on both sides of the neck. Intradermal injection consisted of 0.2 ml of the SK-SD antigen (100 U of streptokinase and 25 U of streptodornase). Seven ponies that had shown strong dermal responses were used in this study. Sixty days after the skin test, prednisolone was administered into four of these ponies, and 4 h later the antigen SK-SD was injected intradermally in the same amount as before on the opposite side of the neck. In addition, from two of the prednisolone-treated ponies, the leukocytes were separated from blood samples collected at 24 and 0 h prior to treatment and 4, 7, 24, and 144 h post-administration. These leukocytes were subsequently used for antigen-induced blast transformation assay. The three remaining ponies served as controls. They were skin tested as above after the administration of 100 ml of saline. The skin thickness was measured at 0 h (before injection) and 24, 48, and 72 h post-injection by caliper.

Statistical analysis. The different types of circulating leukocytes, disintegrations per minute in stimulated and nonstimulated culture, and dermal response before and after administration of the prednisolone were expressed as the arithmetic mean with the standard error. The significant difference between (PHA or SK-SD) stimulated and nonstimulated cultures of the six different sampling periods were determined by split-plot analysis of variance, where stimulation and time were considered as separated factors (19). The Student t test was employed in the determination of significant differences in the dermal response.

RESULTS

Effect of the prednisolone on the leukocyte count. The blood samples were collected at 24 and 0 h preinjection and 2, 4, 7, 24, 48, 72, and 144 h postinjection of the prednisolone. The present study demonstrated that the single intravenous dose of prednisolone has a profound effect upon circulating leukocytes in the eight ponies. The absolute number of circulating lymphocytes rapidly decreased and showed the maximal depression at 7 to 24 h postinjection (Fig. 1). Afterward, their number increased, and, during the next 48 h, they reached the pre-administration level. Similar effect was ob-
The maximal depression of the eosinophils was extended up to 48 h in this case. In contrast, the number of polymorphonuclear cells increased during the first 7 h postinjection and then declined up to 48 h (Fig. 1). There was no marked change in the number of monocytes up to 24 h postinjection. Their number then increased and reached a peak at 48 h. The variation of the number of polymorphonuclear cells and lymphocytes in the three ponies treated with saline was from +6 to −5% of the initial value.

Effect of the prednisolone on the response of the lymphocytes to PHA or SK-SD stimulation. The lymphocytes were separated from blood of the eight ponies which was collected at 24 and 0 h preinjection and 4, 7, 24, and 144 h postinjection of the prednisolone. These lymphocytes were incubated with PHA and their blast transformation was measured by incorporation of \([^3]H\)thymidine. The data of this study demonstrate that blood lymphocytes from ponies responded significantly to the PHA stimulation. The treatment of ponies with prednisolone did not influence the response of the lymphocytes to PHA stimulation (Table 1). The data were also analyzed by comparing the ratios of the disintegrations per minute of stimulated to unstimulated cultures at each sampling period. Table 1 shows the mean of such ratios which are derived from the individual ratios. There were no significant differences among these ratios in the six sampling periods.

The unfractionated leukocytes were separated from blood of the two SK-SD-sensitive ponies which was collected at 24 and 0 h preinjection and 4, 7, 24, and 144 h postinjection of prednisolone. Subsequently, blood leukocytes incubated with SK-SD and their blast transformation was measured. There was slightly less incorporation of \([^3]H\)thymidine into deoxyribonucleic acid of lymphocytes collected at 4 and 7 h after prednisolone injection, and average ratios for each of these sampling periods were, respectively, 1.8 and 2.1. However, this decrease was not significant when compared with ratios of other sampling periods, which ranged from 2.4 to 3.6.

Effect of the prednisolone on the dermal response. Four SK-SD-sensitive ponies were intravenously injected with prednisolone and 4 h later were skin tested. Skin reaction has been observed to the greatest extent at 24 h after the intradermal injection. The dermal response was markedly reduced after administration of the prednisolone (Fig. 3). In the three control SK-SD-sensitive ponies injected intravenously with saline, the dermal response did not significantly change.

DISCUSSION

This study has shown profound effects of intravenous injection of prednisolone on the counts of different leukocytes of ponies. The number of lymphocytes and eosinophils markedly decreased within a few hours after injection. The decrease in number of eosinophils in circulation may be interpreted as the sequestration of eosinophils in tissue (1). There are several reports that corticosteroid treatment has caused lymphocytopenia in rodents (6), horses (14), and humans (8, 23). However, the mechanism for this decrease in the number of cells is still unclear. It seems that the sequestration of the lymphocytes, either in the bone marrow or in the lymphoid organs as in rodents, is more likely than lympholysis. Recently, the

![Fig. 1. Effect of prednisolone administration into the ponies on circulating polymorphonuclear cell (●) and circulating lymphocyte (□) counts. Each point represents mean counts ± standard error of eight ponies for the various times before and after prednisolone injection.](http://iai.asm.org/)
selective depletion of lymphocytes from circulation after corticosteroids treatment has been shown. Yu et al. (23) have shown that human T lymphocytes are more sensitive to the treatment with corticosteroids than human B lymphocytes. In addition, the human concanavalin A-responsive subpopulations of the T lymphocytes are more sensitive to the corticosteroid treatment than the PHA-responsive subpopulation of the T lymphocytes (8).

In contrast, the count of neutrophils increased after administration of prednisolone. This effect is due to the release of the number of neutrophils from a so-called marginal granulocyte pool which normally comprises over half of the leukocytes (20). Present results have also shown that the number of lymphocytes and neutrophils returned to the initial counts within 72 h. Subsequently, mild lymphocytosis and neutropenia were observed.

The results of this study, and those of Osbaldiston and Johnson (14) in horses and Paape et al. (15) in cows did not show significant changes in the number of monocytes after intravenous administration of cortisone. Despite these observations, the dramatic depletion of circulating monocytes after administration of hydrocortisone has been reported (8, 23) in humans.

The ponies' blood lymphocytes responded very well to the stimulation with PHA in vitro. The lymphocytes incubated with PHA incorporated about 14 times more [3H]thymidine than nonstimulated control lymphocytes. This response varied among animals. Similar observations were reported by other investigators working with lymphocytes of humans (10) or rodents (9). The data of this study have also demonstrated that intravenous administration of the prednisolone did not change the response of lymphocytes to the PHA stimulation. These results seem to be consistent with the hypothesis that the PHA-responsive lymphocytes are corticosteroid resistant.

Table 1. Effect of prednisolone administration on the stimulation of lymphocyte transformation with PHA measured by [3H]thymidine incorporation

<table>
<thead>
<tr>
<th>Determinants</th>
<th>[3H]thymidine incorporation into lymphocytes collected at:*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Lymphocytes stimulated with PHA</td>
<td>26,695 ± 2,257</td>
</tr>
<tr>
<td>Lymphocytes non-stimulated</td>
<td>2,301 ± 250</td>
</tr>
<tr>
<td>Index of stimulation*</td>
<td>11.6</td>
</tr>
</tbody>
</table>

* Results shown as the mean of disintegrations per minute ± standard error.
* Index of stimulation is disintegrations per minute of stimulated lymphocytes per disintegrations per minute of nonstimulated lymphocytes.

There is some evidence that corticosteroid treatment of individuals has suppressed the blast transformation of sensitive lymphocytes after stimulation with specific antigen (8). Fauci and Dale have correlated this suppression with the resultant depletion of the number of monocytes in the blood after corticosteroid treatment (8) since antigen-induced blast transformation requires two types of cells, sensitive lymphocytes and monocytes (16). In contrast, the limited results of the present study have indicated neither a significant suppression in blast transformation of SK-SD-sensitive lymphocytes stimulated with the specific antigen, nor a depletion of monocytes in the blood of ponies treated with corticosteroids. It is possible that a larger dose of corticosteroids than was used in the present study would affect the number of circulating monocytes and subse-
dently blast transformation. In addition, Davies and Carmichael (7) have observed that antigen-induced blast transformation rose slightly during 5 days of corticosteroid treatment of calves and after was approximately three- to 12-fold higher than the average pretreatment value. They have also observed no consistent changes in the number of monocytes during and after the treatment. In vitro observations on the effect of corticosteroids on antigen-induced blast transformation are very limited, but it seems that small doses of corticosteroids produced relatively minor effect on antigen-stimulated lymphocyte blast transformation (2, 11). Results of in vitro and in vivo studies have suggested that small doses of corticosteroids do not directly affect antigen-induced blast transformation. However, depletion of monocytes from circulation may be the cause of the suppression of blast transformation (8).

The results of this investigation have shown that the dermal response to the antigen SK-SD was markedly decreased after administration of the prednisolone into SK-SD-sensitive ponies. Similar observations were reported by Bovornkitti et al. (5) that the dermal response to the antigen is diminished in the individuals treated with corticosteroids. Histopathological examination of the delayed skin lesions has revealed that administration of corticosteroids into tuberculin-sensitive guinea pigs prevented the accumulation of macrophages in the lesion to a greater degree than it affected accumulation of small lymphocytes (22). In vitro studies have partially elucidated this suppressive mechanism of corticosteroids in the delayed hypersensitivity reaction. Corticosteroids do not inhibit specifically stimulated lymphocytes to produce lymphokine (2, 21) but do prevent the target cells from responding to macrophages aggregation factor (21) or macrophage migration inhibitory factor (2). However, the mechanism by which corticosteroids inhibit the action of the lymphokine on its target cells is still to be solved.

LITERATURE CITED