# Effect of dexamethasone and equine rhinopneumonitis virus on adrenocortical and immunological responses of foals



by

William David Wilson

A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Veterinary Clinical Sciences
Major: Veterinary Clinical Science
(Veterinary Medicine)

Signatures have been redacted for privacy

Iowa State University
Ames, Iowa

1980

TABLE OF CONTENTS

# I. INTRODUCTION AND OBJECTIVES OF THE STUDY

Infection of horses with equine rhinopneumonitis virus (ERV) has long been associated with respiratory disease in young animals (14,26,59,170) and abortion in pregnant mares (26,44,59,170). In recent years, two additional syndromes have been described with regard to ERV infection; namely a neurologic disorder with various manifestations (20,40,102,103,131, 132,187) and a condition characterized by death of foals during the early neonatal period (14,26,36,57,170). It has been suggested that this latter syndrome is the result of infection of the fetus with ERV late in gestation. Most of the foals so affected die from a variety of bacterial infections and have underlying pathologic lesions which include destruction of the major splenic lymphocyte population and thymus atrophy, or massive necrotic destruction of thymic lymphoid elements, which results in a pronounced peripheral lymphopenia (36). Since aborted fetuses also often have similar necrotic lesions in lymphoreticular tissues (14,44), the suggestion has been raised that this late gestational infection of equine fetuses with ERV induces an immune deficiency state that may prove fatal in early neonatal life. Alternately, if the lymphoid destruction is less severe, foals may survive for longer periods before succumbing to the effects of infection with a variety of microorganisms (36).

The major goal of this research was to investigate the clinicopathologic effects of infection with virulent and attenuated ERV and to establish whether the virus does indeed possess the potential to induce an immune deficiency state in young foals. The risk of inducing abortion by inoculating pregnant mares with virulent ERV late in gestation was considered to be too great to attempt this approach to the problem. Instead, the experimentation was conducted on foals soon after birth in an attempt to mimic the prenatal situation.

It is well-recognized that adrenal glucocorticosteroids and their synthetic analogs have wide ranging effects within the body (23,82,213). One such important action is suppression of the normal immune response through inhibition of inflammation (30,51,82,213), reduction of the number of circulating lymphocytes and eosinophils (6,140,167,205,206),

and inhibition of a number of other important resistance mechanisms such as cell-mediated immunity (79) and interferon (81,116). These effects have been noted in a number of species and are known to increase the susceptibility to infections (51,151,213), cause recrudescence of latent infections (53,192) and magnify the severity of pre-existing infections (43).

In light of the above reports, it appears likely that chronic administration of a potent glucocorticosteroid such as dexamethasone would suppress the body resistance mechanisms of newborn foals and possibly enhance replication of inoculated ERV in lymphoid tissues. In these sites, ERV could exert a destructive effect if it possesses this capability.

Several workers have evaluated adrenocortical function in adult horses by measuring plasma levels of cortisol and other glucocorticoids (28,97,105,124,229), but investigation of the adrenal output of young foals has not been well-documented. As noted above, glucocorticosteroids (GCS) are thought to influence a number of body defense mechanisms. There is good evidence that endogenous GCS levels are high in the neonatal calf and may predispose this species to infectious disease during early life (68). This interaction of adrenal output with immune function may also be of significance in neonatal foals. Such an evaluation was approached in this investigation by the measurement of plasma cortisol concentrations in young foals along with several markers of cell-mediated and humoral immune function. In addition, the influence of exogenous glucocorticoids on these indicators of immune function could be determined.

It is well-documented that the administration of exogenous glucocorticosteroids reduces adrenal output in a number of species (18,97, 105,113,201), and that long-term therapy with these agents may lead to the development of an adrenocortical insufficiency which becomes manifest when such therapy is terminated (88,178). It has been speculated that the "Turning out syndrome", which occurs in adult performance horses given frequent doses of glucocorticosteroids during the racing season,

is a manifestation of this iatrogenic hypoadrenalcorticism (118,164, 198). However, no controlled quantitative studies of this effect have been performed in the horse, and thus an additional goal of this study was to determine the influence of chronic DXM administration on the adrenal output of young foals.

The evaluation of blood samples for such parameters as leucocyte count, total protein concentration, and plasma fibrinogen concentration is frequently conducted to assess the nature of disease states affecting an animal and the response of the animal to such diseases. Ranges of "normal" values have been determined for adult horses (190), but not for pony foals. Therefore, it was necessary to establish values for these hematological parameters in order to accurately evaluate the effects of DXM and ERV treatment on these important markers of body defense in the neonatal equine.

Juelsgaard (112) reported that foals as young as two weeks of age possess very mature humoral immune function and respond well to vaccination. A further objective of this research was to extend the investigation of the serological responses of foals into the first few days of life, and, in addition, evaluate the cell-mediated component of the immune system of young foals.

# II. LITERATURE REVIEW

## A. Plasma Adrenal Glucocorticosteroids

### 1. Basic physiology

Cortisol and the other major natural glucocorticosteroids (GCS) are predominantly secreted by the inner two zones of the adrenal cortex, namely the zona fasciculata and zona reticularis, which contain the necessary inductive enzymes (84). This secretion is the end result of a complex neuroendocrine pathway which involves the hypothalamus, pituitary and adrenals (84,145,173).

Neural inputs converge on the medial eminence of the hypothalamus, to control the secretion of corticotrophin releasing factor or factors (CRF). These inputs may be either inhibitory or excitatory, the latter including various forms of stress such as pain or trauma, emotional reactions such as fright or anxiety, and other influences including pyrogens and hypoglycemia. It is thought that release of CRF is stimulated by cholinergic fibers and inhibited by adrenergic neurons. In addition, the CNS imposes a diurnal cycle on CRF release, being elevated in the early morning and decreasing to a low in the evening (173). CRF passes via the hypophyseal portal vessels directly to the anterior pituitary where it stimulates adrenocorticotrophic hormone (ACTH) secretion (84,173).

The released ACTH passes directly into the systemic circulation by which it reaches the adrenal cortex where it stimulates production and release of cortisol and other glucocorticoids from the zona fasciculata and zona reticularis. These areas appear to display a diurnal sensitivity to ACTH in man (84). ACTH is the sole hormone responsible for adrenal release of GCS (145) and, in addition to this function, it maintains cortical integrity and sensitivity to further ACTH stimulation (84,173).

Increased plasma GCS concentrations stimulate a negative feedback loop which restricts further release. GCS inhibition is thought to act

by suppressing pituitary ACTH secretion (84,173), but now there is good evidence to suggest that hypothalamic formation and release of CRF is also retarded (173). Furthermore, excitatory neural impulses appear to be able to override this normal regulatory mechanism since, in conditions invoking severe stress, high ACTH secretion and cortisol output occur in the presence of elevated GCS levels (84).

## 2. Assay procedures

During the last thirty years, a number of assay procedures have been developed to measure plasma GCS concentrations in man and animals with the hope of characterizing normal adrenocortical function and deviations therefrom.

The Thorn procedure (210), which measured ACTH-induced depression of circulating eosinophil counts, was not quantitative and gave little indication of normal endogenous function but was of some use in the confirmation of adrenocortical insufficiency. Porter and Silber (171) and Silber and Porter (194) reported the first quantitative assay of adrenal steroids. This method depends on the reaction of 17-hydroxycorticosteroids with phenylhydrazine in sulfuric acid to produce a yellow color which can be measured colorimetrically. This procedure is said to be relatively specific for cortisol (201), but it also measures additional 17-hydroxycorticosteroids such as cortisone, 11-deoxycortisol, and others and is subject to interference by contaminants (175). In addition, the method lacks sensitivity for low values (194) and requires large volumes of plasma and time-consuming extraction steps for good results.

A fluorimetric technique developed by Mattingly (144) has proven relatively simple and easy to perform on small, crudely extracted, plasma samples. The method depends upon the fluorescence of 11-hydroxycorticosteroids in ethanol-sulfuric acid and consequently it measures both cortisol and corticosterone (144,156,201). This does not pose a problem in samples with low corticosterone concentrations, but since the latter gives a fluorescence two and one-half times that of cortisol, overestimation of cortisol values frequently occurs (90,111,125,156,201).

Furthermore, other steroids such as estrogens and cholesterol fluoresce, as do nonsteroidal substances in both plasma and the reagents, further compromising specificity (38,111,175). Quenching can also occur, especially in icteric plasma (144), and the interval between mixing of the reagents and reading the fluorescence is critical (144). The method also lacks good sensitivity for low values (45,125,129,144), a factor which limits its use in domestic animals.

A double isotopic dilution (DID) technique, involving the introduction of two radioactive isotopes into the steroid molecule with subsequent comparison of their ratios, has been found to be sensitive and specific but is too time-consuming and costly for routine use (175,201).

The majority of cortisol determinations are currently performed using radioligand procedures, namely competitive protein binding (CPB) (157) and radioimmunoassay (RIA) (75). Both depend upon the competitive equilibration of cortisol in the sample with added labeled cortisol for binding sites on protein molecules. In the case of CPB, this protein is corticosteroid binding globulin (CBG) whereas, in RIA, it is a specific anti-cortisol antibody. The ratio of free to bound labeled steroid is then determined by precipitation and separation of the bound fraction followed by counting of the associated radioactivity. This ratio can then be compared to that of known calibrators on a standard curve and the cortisol content of the sample calculated (1).

The inherently greater specificity of antibody than CBG for cortisol means, in general, that RIA procedures are more sensitive, specific, and accurate than CPB assays, but both are superior to the previously outlined methods and require only a small reaction volume (111). The major drawback with the CPB procedure is that CBG will bind all 11-hydroxycorticosteroids especially corticosterone (90,111,156), as well as other natural and synthetic steroids including progesterone and prednisone (111, 201), and thus chromatographic or solvent extraction steps are needed to increase the assay performance. While this was also the case for most early RIA methods, recent developments have allowed elimination of the time-consuming extraction step without compromising validity (125).

Many variations of the basic RIA technique have been reported in recent years (32,61,71,75,113,125,211) and relate mainly to the method for freeing cortisol from endogenous CBG, the specificity and affinity of the first antibody, the nature of the radio-tracer and the method used to precipitate cortisol-antibody complexes. Detailed examination of these principles is beyond the scope of this discussion, but the subject has been excellently reviewed by Abraham (1) and Jeffcoate (106). In general, however, it is relevant to note that antibodies obtained by immunization of animals with cortisol conjugated to the carrier protein at the 3-position of the steroid nucleus, are more highly specific than those produced when 21-position conjugates are used (74). In addition, $^{125}I$-labeled tracers appear to be superior to those with $^{3}H$ labels (106) and a double antibody precipitation procedure has many advantages over charcoal absorption and other separation techniques despite the increased expense and time involved (106).

Irrespective of the assay method used, it is imperative that each investigator validate his procedure on the basis of sensitivity, specificity, precision and accuracy (89).

3. Nature of plasma steroids

The first truly quantitative and qualitative study of equine endogenous adrenal steroids was performed by Zolovick et al. (229) who found cortisol to be the major plasma glucocorticosteroid (GCS). Lesser amounts of cortisone, corticosterone and deoxycorticosterone were also present such that the cortisol:cortisone:corticosterone ratio was 16:8:0.5. Subsequently, plasma cortisol: corticosterone ratios of 6 to 8 :1 (28) and 14:1 (105) were reported in domestic horses and ponies as well as a ratio of 168:1 in wild horse stallions (119). The finding of high cortisone concentrations in the initial report (229) has not been confirmed. In bovine plasma, cortisol:corticosterone ratios of 1.5 to 4:1 occur, depending on the breed and age (176,219), with cortisone present in lesser amounts (185). Similar ratios have been observed in pigs (28) and dogs (84,90,111) and cortisol has been identified as the major circulating GCS

in sheep (15,49,185,207), man (95,113,120), monkeys (147), and guinea pigs (52), whilst corticosterone predominates in the rat (64,148). In human neonates, umbilical cord and peripheral plasma show relatively higher concentrations of cortisone than cortisol (95,120) with significant amounts of corticosterone, cortisone sulfate and 11-deoxycorticosterone sulfate also present (120).

4. Resting adult levels

Plasma GCS concentrations have been measured in ponies and domestic and wild horses using fluorimetric (105,229), CPB (28,96,97,119,160,182) and RIA (124) procedures. Such a diversity of assay techniques makes comparison of the results of different investigations difficult, but, with the exception of one report (229), published values are relatively consistent.

Zolovick et al. (229) observed a diurnal variation, in the total plasma GCS levels of stabled horses, with a 10 am peak of 395.3 µg/dl and a 10 pm low of 219 µg/dl. A much lower normal range of 3-13 µg/dl was quoted by James et al. (105) in ponies, using a similar fluorimetric assay procedure. Cortisol levels remained relatively stable during the morning (mean 7 µg/dl) but fell in the afternoon reaching 68% of this level at the 9 pm nadir. Short-term oscillations were common but the overall trend, of increase during the night with peaks in the morning and a fall during the rest of the day, was maintained. Marked inter-individual variations were noted but tended to remain consistent from day to day.

Hoffsis et al. (97) reported mean total plasma GCS levels of 5.1 µg/dl in resting thoroughbred, standardbred and quarter horses using a CPB assay. In spite of marked individual variation from day to day, an overall diurnal pattern was evident with maximum and minimum values at 8 am and 4 pm, respectively. A lower mean daily concentration of 1.37 µg/dl was observed in mixed-breed ponies in the investigations of Bottoms et al. (28). The diurnal pattern was sinusoidal in character, peaking at 5-10 am, with low values in the evening. Similar levels and trends were noted by Nathanielsz et al. (160), employing the same CPB technique, in

late gestation Shetland mares. Kumar et al. (124) have published the only RIA-derived equine plasma cortisol values. Ponies maintained on a strict twelve hour, 6 am to 6 pm, lighting schedule showed a diurnal cycle characterized by maximum concentrations at 9 to 11 am (3.2 µg/dl), with minimum values twelve hours later (1.9 µg/dl).

Kirkpatrick et al. (119) noted mean daily plasma corticoid levels of 4.0 µg/dl in captive wild horse stallions. The diurnal trends, however, differed from those of domesticated animals in that no morning peak occurred after the 11 pm nadir; instead it was replaced by a high plateau between midnight and 5 pm.

Cattle appear to have a lower overall range of cortisol values than is seen in horses, although diurnal trends are similar (72,134,220). Mean daily concentrations of 0.5 µg/dl to 2.0 µg/dl are considered normal (47) but levels from 0.5 µg/dl to 7.3 µg/dl have been derived by various assay techniques (47,58,134,185,220). In sheep, resting mean daily cortisol values of .1 µg/dl to 3.8 µg/dl have been noted (15,45,62,98, 137,185) and the accepted normal range is similar to that of cattle. Multiple peaks and troughs of secretion have been observed in sheep during the hours of daylight but an overall diurnal pattern, similar to that seen in the horse, prevails. Very low plasma concentrations during the period of darkness may indicate that little or no cortisol secretion occurs for up to 2/3 of the twenty-four hour cycle (137,138).

Normal dogs exhibit a diurnal pattern of cortisol secretion and reported values are 1.6 to 6.8 µg/dl using fluorimetric assays (31,38,90), 1.3 to 5.0 µg/dl with CPB procedures (84,201) and 1.78 to 2.3 µg/dl for cortisol-specific RIA's (19,41,111). The wide range of plasma GCS concentrations noted in pigs (28,67,117,130,215,225) likely refects the difficulty in obtaining unstressed samples. However, blood collection via jugular catheters followed by a CPB assay gives a mean daily range of 1 to 2 µg/dl with a distinct diurnal variation, equivalent to that seen in horses, being evident in young pigs (28,225), sows (117), and boars (130).

Plasma GCS concentrations in primates are, in general, higher than

those of domestic animals but equivalent diurnal trends are usually apparent. Colorimetric determinations of corticoids in monkeys have revealed minimum daily levels at 9 pm to be 60% of the peak daily concentration of 56 µg/dl seen at 6 am (147). Morning plasma GCS levels of 5-30 µg/dl are recognized as being normal in man and many workers, using a variety of assay techniques, have reported values within this range (39,71,113,129,144,178,194). A great deal of variation within individuals on the same day and between individuals on different days has been reported (91). However, a characteristic diurnal variation with morning values significantly higher than afternoon and evening values is the rule (39,61,71,80,113,125,126,127,129,145,166,211). Minimum concentrations occur during the early hours of sleep with a rapid regular rise during the remainder of sleep, reaching maximum levels at the time of awakening, followed by a fall during the working day (166). Evening cortisol levels should be less than 50% of the morning value in normal subjects (129,145,211).

Orth et al. (166) showed that the diurnal pattern observed in man was a reflection of the habitual sleep-wake cycle rather than being related to the twenty-four hour day length. Alteration of the cycle for a single day by either prolonging or abruptly terminating sleep, did not alter the established diurnal rhythm. However, consistent alterations of sleep-wake patterns for seven days gave new diurnal periods corresponding to the new cycles of 12, 19, and 33 hours respectively. Another investigator showed that a subject who habitually slept from 4 am until noon showed a shift in the cortisol secretion pattern to match this sleep schedule (126). Babies have sleeping cycles much different from those of adults and do not have diurnal cortisol rhythms even at four months of age. Whilst these gradually develop they do not equate with the character of the adult cycle until children are 1-3 years old, further confirming that the adult diurnal pattern is an acquired, rather than built-in one (80).

In contrast, the diurnal rhythm in sheep seems to be related to the light/dark cycle, since if sheep are acutely exposed to lighting for an

entire twenty-four hours, the diurnal pattern is lost (137).

5. Effect of age and sex

Hoffsis et al. (97) were unable to find any age effects on corticoid levels in horses between one and seventeen years old. A similar situation appears to exist in cattle, although aging bulls seem to have a reduced responsiveness to ACTH and may require higher plasma ACTH levels to maintain a constant plasma GCS concentration (176). No apparent age effect was observed in one investigation of cortisol levels in human subjects 15-76 years old (144), although others have reported decreasing GCS concentrations with advancing age (25).

Rossdale et al. (182) demonstrated a significant increase in plasma cortisol concentrations, from 6.8 to 8.3 µg/dl, between ten and thirty minutes after birth in thoroughbred foals. After remaining stable for three hours, a precipitous drop occurred with levels halved at six hours of age. A similar pattern was found in newborn pony foals (182). Analogous peaking of cortisol levels followed by a rapid fall during the first twenty-four hours of life has been noted in cattle (47,68,101) and sheep (16,62,159,207). In calves, concentrations continue to decline over the first twelve days of life before stabilizing (68,101). Peak cortisol levels immediately after birth in sheep (16,62,207) and cattle (47,68,101) are higher than those seen in foals (182), probably due to the absence of the spectacular late gestation fetal cortisol surge which occurs in the former two species (160).

Hillman and Giroud (95) and Klein et al. (120) found cortisone to be the major circulating GCS in newborn infants and it was still a significant component at two weeks of age. Total GCS levels were three times greater in newborns than they were in adults (120) but fell during the first two weeks of life, especially in the initial forty-eight hours. One to five month old infants had total GCS levels and cortisol:cortisone ratios equivalent to those of adults (95), but mature diurnal rhythms were absent until one to three years of age (80).

Premature foals have much lower cortisol levels than those born at

full-term and lack the characteristic rise and fall during the early postnatal period (160,182). Similarly, premature human infants have reduced total GCS concentrations, due largely to lowered cortisone levels (120).

Significant sex differences in plasma GCS values have not been evident in horses (97,105), pigs (67), dogs (19,31,41,111), man (113,120, 125), or monkeys (147). Levels do not appear to be altered during pregnancy in horses (160,182) or cattle (134) but pregnant women develop cortisol concentrations two to three times higher than those of non-pregnant women (113,125) and a similar pattern is seen in gravid rats (148). This finding is thought to be due to the increased plasma concentrations of corticoid binding globulin present during pregnancy, at least in man (70).

6. Effect of exercise, stress and disease

Racehorses in training develop a 30% increase in cortisol levels after a normal exercise gallop (105) and standardbred racehorses have higher concentrations after exercise and before racing than do resting horses of similar type (97). Young men show a transient elevation of cortisol levels, in the neighborhood of 70%, following heavy bicycle exercise but not after lighter work, indicating that less than maximal exercise does not severely stress the individual (93).

de Lacerda et al. (126) showed that irregular increases in human plasma cortisol levels during the normal working day were closely related to psychological stresses such as frustration and anxiety. An analogous situation is seen in wild horses (119) and pigs (67,215) which exhibit markedly elevated plasma concentrations in response to the stress of handling and restraint. Similarly, sheep placed in a new environment lose their diurnal cortisol rhythms and have elevated mean daily values during the period of adaptation (98,138).

Untrained sheep experienced a substantial elevation in cortisol levels due to the stress of venipuncture, but no significant effect was noted in samples collected one hour after venipuncture in trained sheep

(15). While venipuncture does not appear to affect cortisol values in horses (97,105) or cattle (191) at the time of sampling, repeated jugular bleeding at two hour intervals produced altered concentrations in later samples obtained from horses (97). However, the half-life of cortisol in the horse has been estimated to be 80 minutes (105) and thus the effect of venipuncture on samples taken twelve to twenty-four hours later should be negligible, as it is in man (70).

James et al. (105) reported a marked rise in GCS levels in horses subjected to surgery, the greatest increases occurring during the longest procedures. Similar observations have been made in pigs (117), sheep (16,45), man (115), and dogs (19,84). In dogs, ACTH levels rise within one minute of the initiation of the surgical procedure and the increase in plasma corticoids lags 4 to 5 minutes behind (84). With moderate to severe surgical stress, canine ACTH levels often exceed those needed for maximal adrenal stimulation (84) and a similar situation probably occurs in man (115). After peaking at four to five times resting levels three hours into a surgical procedure, levels fall to near normal by twenty-four hours in man (115) and also in dogs, irrespective of the duration and severity of the procedure (38). In contrast, sheep maintain elevated cortisol levels for several days following surgery (16).

Many disease states appear to stimulate increased adrenocortical output with a subsequent elevation in plasma GCS levels. Extremely high cortisol concentrations have been noted in premature infants with respiratory distress syndrome (120) and hyaline membrane disease (174); the highest values being seen in fatal rather than benign cases (174). Full-term foals exhibiting postnatal convulsions and lactic acidosis maintained markedly elevated levels of cortisol in spite of pharmacological modulation of muscular activity (160,182). Similarly, calves which developed infectious diarrhea had higher concentrations, both before and after signs appeared, than did healthy calves (101,133) and those which died showed a greater elevation than those which recovered (133). Cortisol levels remained elevated up to the time of death indicating that calves are able to respond to the severe stress of fluid and electrolyte imbalance

and diarrhea without terminal adrenocortical failure (133).

Hoffsis and Murdick (96) indicated that the pathological conditions which produced the most marked adrenal stimulation in horses were acute illnesses such as fractures, colic, shock and dystocia with levels of up to 43 µg/dl occurring in animals with fractures. A similar situation appears to exist in man where very high cortisol concentrations have been observed in patients with unbalanced diabetes, severe cardiac infarction, acute bacterial infections, severe trauma, shock, tetanus, meningitis, and other acute conditions (104,121,129,180). Human intensive care patients subject to prolonged stress experienced pronounced adrenocortical stimulation as indicated by markedly increased cortisol secretion rates up to double those seen in patients with established Cushing's Disease (121). Furthermore, the very high cortisol levels in children with meningitis could not be further increased by ACTH administration, indicating that the adrenals were already maximally stimulated (180). Another prominent early alteration found in patients with acute diseases was a loss of the normal diurnal rhythm. Morning values were often within normal limits but no afternoon decline occurred (129,180); a further change typical of hyperadrenalcorticism (19,23).

Jacobs and Nabarro (104) suggested that elevation of plasma corticoid concentrations was a natural reaction of human patients to the stress of infection, and considered the adrenal response to be deficient if levels remained less than 15 µg/dl. They further speculated that elevated values represent deficient hepatic metabolism in addition to the increased adrenocortical secretion in response to hypothalamus stimulation. The fact that human subjects with chronic or terminal illnesses have very high plasma cortisol levels (104) seems to support this view. In contrast, horses with chronic debilitating disease conditions may experience some degree of adrenal exhaustion, as evidenced by lower than normal circulating GCS concentrations (96).

## B. Effect of Exogenous Glucocorticosteroids on Adrenal Output

The increased use of glucocorticosteroids (GCS) in equine practice in recent years has led to the belief that prolonged therapy with such drugs is the major cause of secondary adrenocortical insufficiency in the horse (118,164); a condition commonly referred to as the "turning out syndrome" (197). It is now well-established that administration of exogenous GCS can suppress endogenous plasma corticoid levels in normal horses (96,97,105, 173), man (88,113,125,145,155,162,178,211), dogs (84,90,111,201), cattle (47), sheep (18,45), and rats (64). However, the chronicity of administration appears to markedly influence the duration and severity of effects observed (88).

### 1. Acute administration

James et al. (105) noted a steady fall in plasma corticoid concentrations following intravenous injection of a pony with dexamethasone (DXM). This decline was exponential in character over the first three hours, with a half life of 150 minutes, and levels were still depressed twenty-four hours postinjection. Hoffsis et al. (97) further demonstrated that even small doses of DXM could maximally suppress adrenocortical secretion in adult horses. Reduced plasma corticoid levels were evident by one hour after intramuscular administration of two to eighty mg of DXM and maximal suppression, as indicated by values less than 1 µg/dl, occurred between twelve and twenty-four hours, followed by a return to normal by seventy-two hours postinjection. A similar duration of suppression was noted by Ray (173) who also showed that short-term DXM treatment, even with high doses, did not depress the adrenal response to exogenous ACTH.

An equivalent, profound but transient, depression of cortisol concentrations has been observed in sheep (18,45), and Beaven (18) noted complete cessation of adrenocortical secretion within one hour of DXM injection. Cortisol levels of less than 0.5 µg/dl have been seen in dogs within one hour of intravenous DXM treatment (201) and undetectable values

persisted for at least thrity-two hours after a single oral dose (111). In canine (90,201) and human (113,125,145,211) medicine, acute suppression of plasma corticoid levels is used as a clinical test to assess the normality of adrenocortical function. A normal response in human patients is indicated by cortisol concentrations of less than 5 µg/dl (2 µg/dl using RIA) eight to ten hours after oral administration of DXM (113,125, 145,211), with return to pretreatment levels within twenty-four to forty-eight hours (178).

The inhibitory effects of exogenous GCS on adrenocortical secretion may, in some cases, be overridden by various stresses. This effect was demonstrated by a delayed depression of cortisol concentrations following surgical stress in dogs maintained on a continuous intravenous DXM drip (84). Similarly, higher doses of DXM were needed to suppress cortisol levels in stressed sheep than in unstressed sheep (18). Ganong et al. (84) suggested that during stress the inhibitory effect of exogenous steroids was balanced in some way by an increased excitatory input to the ACTH-secreting mechanism.

These suppressive effects also appear to be influenced by the time of DXM administration in relation to the diurnal cycle; the greatest and most prolonged inhibition occurring when exogenous steroids are given four to eight hours before the expected peak endogenous levels (64,162). It has been suggested that once the early morning peak of ACTH release is suppressed, it remains so until the next natural rise the following morning, even though no more DXM is given. In addition, the central mechanism controlling ACTH release may require higher corticoid levels for suppression during the early morning, when secretion is normally maximal, than at other times during the day (162).

2. Chronic administration

Controlled studies regarding the effects of prolonged GCS administration on adrenocortical function in the horse are few. Hoffsis and Murdick (96) intramuscularly administered 20 mg daily doses of DXM to three adult horses for an extended period and observed sustained depression of

plasma corticoids. Evaluation of the pattern of recovery was impeded by the death of two of the animals but, while one horse developed elevated cortisol levels within the normal range prior to death, the other showed no such rise despite the severe stress of thrombo-embolic colic.

Robinson et al. (178) observed complete inhibition of adrenal activity in human patients maintained on exogenous steroids for periods of one to eighteen months. That the pituitary-adrenal axis did not suffer permanent damage was indicated by the rapid rise in cortisol levels observed by twenty-four hours after sudden cessation of treatment. Plasma concentrations were normal at forty-eight hours as were cortisol secretion rates five days after withdrawal. A similar investigation by Morris and Jorgensen (155) indicated that asthmatic children, on steroid therapy for a mean period of seven years (minimum six months), rapidly recovered from the sustained and complete suppressive adrenal effects when treatment was discontinued. Reduction of the daily prednisone dose was inversely correlated with increased adrenocortical responses and within one to two weeks all patients had responses similar to those of nontreated patients.

Good evidence remains, however, that some patients suffer adrenocortical insufficiency states following cessation of chronic GCS therapy. Robinson et al. (178) demonstrated a failure of recovery of plasma corticoid levels to normal, along with signs of acute adrenocortical insufficiency, in one patient after abrupt termination of a continuous three and one-half year course of steroid treatment. Graber et al. (88) observed similar effects after steroid withdrawal in patients which had experienced supraphysiological levels for one to ten years as a result of exogenous administration or functional adrenal tumors. Recovery of normal function followed a definite pattern characterized by four distinct phases. During the first month clinical symptoms of adrenal insufficiency were noted and both plasma GCS and ACTH concentrations were subnormal as was the response to exogenous ACTH, a pattern characteristic of hypopituitarism. ACTH levels rose to above normal during the next four months but plasma corticoids and adrenal responses to ACTH remained subnormal, indicating that the pituitary response to low GCS levels was

now intact but the adrenal response to released ACTH was still deficient. The sixth to ninth months of convalescence were characterized by normal plasma corticoid levels, but the elevated ACTH concentrations remaining in some patients was evidence of continued depression of adrenal responsiveness. Adrenal responses to ACTH subsequently returned to normal.

Other workers have observed defective pituitary release of ACTH (155,178) and reduced pituitary levels of ACTH during and after prolonged GCS therapy in man (99). Furthermore, reduced adrenal responsiveness to ACTH has been noted in horses (96,173) and in sheep (18) whose adrenal secretion was completely suppressed with DXM. However, normal responses were seen in sheep which were incompletely suppressed suggesting that basal levels of ACTH are needed to "prime" this response (18). It also appears that humans (88) and rats (64) subjected to prolonged high circulating GCS concentrations develop true adrenal atrophy resulting in further reduction of the cortisol secreting capacity (155).

The above studies provide good evidence that pituitary ACTH release in response to low plasma corticoid levels and the adrenal response to released ACTH are both inhibited by prolonged GCS therapy. The finding of histological lesions in both limbs of this system (21) adds further credence to this conclusion.

## C. Effect of Glucocorticosteroids on Immune Responses

### 1. General

The recognition of the physiological importance of the adrenal cortex over 100 years ago led to the early therapeutic use of crude adrenal extracts. Later, hormones contained in these extracts were classified as either glucocorticoids, mineralocorticoids or adrenal sex hormones based on their functional characteristics (213). The structures of the naturally occurring adrenal steroids have now been elucidated and the synthesis of analogues with selected pharmacological properties achieved (213).

This review considers the naturally occurring and synthetic glucocorticosteroids (GCS) which are now in widespread clinical use for the treatment of a great variety of conditions (79), based on their effects on a wide range of body functions (23,82,213). These agents are of particular importance in treating inflammatory and immunologically mediated diseases due to their many actions on these systems (43,79, 230). These same actions are responsible for some of the adverse effects encountered following GCS therapy.

The early recognition that infections are more severe in patients with untreated Addison's or Cushing's Disease (114) has led to the belief that the eucorticoid state confers maximal host resistance to infection (23). Recently it has been noted that prolonged systemic use of synthetic GCS render treated subjects more susceptible to a variety of infections (51,151,213). In addition, GCS treatment can cause recrudescence of latent viral infections (53,192) and their use in the face of preexisting infection may lead to increased severity of the condition (43).

2. Influencing variables

a. The agent It is widely believed that the various GCS preparation differ mainly in their relative potency and duration of action rather than in their fundamental effects on the immune system (43). However, so called "equipotent" anti-inflammatory doses of various GCS have been shown to cause qualitative differential effects on lymphocyte populations; dexamethasone (DXM) clearly showing the greatest immunosuppression (78). Thus a degree of caution is needed when comparing results of investigations using different steroid preparations.

b. Dosage rate and chronicity of administration The dosage rate of a particular GCS agent used can markedly affect the immunosuppressive effects observed (30,76,167) and the dosages used in experimental studies often greatly exceed therapeutic recommendations. Similarly, the concentrations of GCS used in in vitro studies are often very high and are seldom

reached by in vivo administration of these agents. These factors may partially explain qualitative and quantitative variations in immunosuppressive effects observed in different studies (140).

However, there does appear to be a maximal degree of suppression possible, at least in the case of peripheral lymphopenia, beyond which increased dosage rates do not cause increased effects (78). The duration of administration of a GCS drug is a factor of major importance in determining the effects observed; chronic treatment giving a wider range and greater degree of immunosuppressive effects than a single dose (11).

c. The species Marked species differences in the immunosuppressive action of GCS exist (43), a factor that has given rise to a great deal of confusion in this regard. These species differences, where relevant, are noted during this review.

3. Effect on blood leukocyte counts

GCS induce a relatively consistent pattern of alteration in the leukograms of a number of species. A leukocytosis with absolute neutrophilia, lymphopenia and eosinopenia is also accompanied by a monocytopenia in some species, including man, but not the horse.

Archer (6) found a pronounced neutrophilia and lymphopenia in horses within four to five hours after administration of a combined dose of ACTH and hydrocortisone. The effect was maximal twenty-four hours after the initial dosage but tended to return towards normal by forty-eight hours despite continued dosing at twelve hour intervals. Peripheral eosinophil counts fell more gradually, reaching their minimum levels forty-eight hours after the first dose before starting to rise again twenty-four hours after the last dose. A transient rise to supra-normal counts was followed by a return to normal at ninety-six hours. Alexander and Ash (2) reported a similar effect after ACTH administration in the horse.

Osbaldiston and Johnson (167) reported that ACTH, DXM, and flumethasone all rapidly induced a significant neutrophilic leukocytosis accompanied by lymphopenia in horses. ACTH and DXM also caused an eosinopenia but none of the agents affected blood monocyte counts. The effects

of flumethasone were generally of lesser magnitude and shorter duration than those of DXM which, in doses of 10-20 mg, produced an increase in neutrophil counts of 96-185% lasting 10-17 hours accompanied by a 41-72% reduction in lymphocyte counts lasting 14-16 hours after a single intramuscular dose. Similar, but more prolonged, effects were noted by Targowski (205) in ponies given a higher (2 mg/Kg) intravenous dose of DXM. Effects were seen at two hours and lymphocyte counts showed a maximal depression of more than 50% between seven and twenty-four hours postinjection. Neutrophil counts showed a three-fold increase peaking at the seventh hour. Maximal eosinopenia extended up to forty-eight hours at which time a transient monocytosis occurred, neutrophil and lymphocyte counts having returned to normal.

Tarr and Olsen (206) showed the above leukogram changes to persist in adult horses during a four day course of DXM treatment at a lower dosage level (approximately .08-.16 mg/Kg). Monocyte counts were unaffected and the persistent neutrophilia, lymphocytopenia and eosinopenia were resolved by thirty-six hours after the last treatment. The results of Magnuson et al. (140), who treated ponies with nine daily intravenous 1 mg/Kg doses of DXM, conflict slightly with the above. After an initial marked increase following the first dose, neutrophil counts fell to below baseline values on the third treatment day, at which time band forms were the predominant cell type. A persistent lymphopenia and slight monocytosis was followed by a return to normal counts by twenty-four hours after the last dose of DXM.

In man, the major leukogram changes, i.e., leukocytosis, neutrophilia, lymphopenia and eosinopenia, are equivalent to those described in the horse. Maximal neutrophilia and lymphopenia occur four to six hours after oral administration of prednisone and DXM (78,228) or intravenous treatment with various doses of hydrocortisone (76). Counts return to normal within 24 hours after a single dose of these agents (76,78,228), the return being slightly faster at lower hydrocortisone dosage rates. In contrast to findings in the horse, however, monocytopenia is a consistent GCS-induced leukogram change observed in man, being even more marked than

the lymphopenia (228). Yu et al. (228) observed an 88% reduction in monocyte counts four hours after oral administration of prednisone, whereas a maximal lymphocyte suppression of 68% was recorded at six hours. A slight rebound in monocyte counts to supra-normal levels has also been noted in man twenty-four hours after GCS administration (78,168). The transient leukogram changes noted above, with return to normal counts by twenty-four hours after GCS administration, have also been observed in patients on alternate day steroid therapy for as long as several years. In this situation the lymphopenia is not cumulative, the kinetics being strictly related to each GCS dose (79).

The pattern of leukogram changes observed in guinea pigs after a single dose of hydrocortisone or methylpredisolone is essentially identical to that observed in man (11,77). The maximal degree of lymphopenia observed during a chronic (seven day) course of cortisone acetate treatment is no greater than that following a single intravenous dose of hydrocortisone (77). However, a lymphopenia persisting at least two weeks after a course of cortisone acetate treatment has been noted, the lymphocyte counts correlating inversely with elevated plasma GCS levels (11).

4. Specific effects on lymphocyte populations

The lymphocyte plays a central role in the specific immune response to antigens (230). It is thought that differentiation of multipotential bone marrow stem cells produces lymphoid stem cells which are processed by either the thymus or bursa equivalent tissues (the primary lymphoid organs). Seeding, via the peripheral circulation, of the resulting lymphoid cells results in population of specific areas of peripheral lymphoid organs such as the spleen, tonsil, lymph nodes and Peyers Patches. The cells initially processed by the thymus are termed "T" cells (small lymphocytes) which, after antigen stimulation, initiate the effector arm of cell-mediated immune reactions (216). The cells processed by the bursal equivalent organs ("B" cells) transform after antigen contact to become plasma cells which produce antibodies, the effector of the humoral immune response (48). The T-cells may also play a role in the humoral response

by active cooperation with B-cells allowing production of antibodies against T-cell dependent antigens (230).

T- and B-lymphocytes and subpopulations of each are differentiated on the basis of physical and functional properties, surface markers and their in vitro response to certain plant mitogens, antigens and other agents (76,77,78,140,168,205,206,228). This heterogeneity of lymphocyte populations may account for their differential responses to GCS (43).

The marked species differences in susceptibility to glucocorticosteroids have led to the broad classification of mammals into either steroid-sensitive or steroid-resistant groups (43). The mouse, rat, hamster and rabbit are considered steroid-sensitive and GCS administration in these species results in a rapid decrease in thymic weight associated with intrathymic cell death, primarily of cortical cells (87). In addition, shrinkage of the spleen and lymph nodes and an associated profound lymphopenia is, in part, due to the lysis of certain lymphocyte subpopulations.

Steroid resistant species include the ferret, monkey, guinea pig, man (43) and probably the horse (139,140). In these species, central and peripheral lymphoid cells are not lysed and are generally less affected by GCS given in vivo or in vitro (43). Extrapolation from studies in different species has led to much confusion, therefore this review will concentrate on steroid-resistant species.

GCS administration causes a marked transient lymphopenia in the horse (6,140,167,205,206). Magnuson et al. (140) quantitated circulating B-lymphocytes by fluorescent antibody staining of surface immunoglobulin and also employed an opsonized SRBC rosette assay for $C_3$ receptor-bearing cells. They found that DXM administration in vivo depleted both B and T-cells, with a proportionately greater effect on T-cells, as indicated by a relative increase in the percentage of Ig-bearing and $C_3$ receptor-bearing cells remaining in the circulation. The T:B-cell ratio returned to normal within forty-eight hours of the last of a series of DXM treatments. In addition, no decrease in lymphocyte viability, as determined by trypan

blue exclusion and $^{51}Cr$ release, was found after *in vitro* incubation with various doses of prednisolone (139,140). It is thus believed that, in the horse, GCS cause redistribution of circulating lymphocytes (139,140,205) and that mature lymphocytes from the spleen and bone marrow repopulate the circulating pool after cessation of steroid treatment (139,140).

Similar studies in man (76,78,168,228) and in guinea pigs (77) have shown that all lymphocyte subpopulations are reduced by GCS, the effect on T-cells again being more marked than the effect on B-cells. Furthermore, Parrillo and Fauci (168) showed that the relative decrease in cells bearing Fc receptors (so called K-cells) was much less than that induced in other lymphocyte subpopulations.

In guinea pigs, it has been conclusively shown that GCS induce redistribution of circulating lymphocytes to the bone marrow (77). A similar situation probably exists in man, since there is no evidence for a lytic effect of GCS on lymphocytes of either species (76,228). The lymphocytes which redistribute to other body compartments under GCS influence are probably the same cells that migrate freely under normal circumstances, namely recirculating, primarily long-lived cells which comprise about two-thirds of the lymphocytes in the intravascular space. The remaining intravascular lymphocytes live out their life in that space, are nonrecirculating and appear to be affected very little, if at all, by GCS (79). Alteration of lymphocyte distribution may be due to the effects of GCS on the vascular endothelium and/or surface changes on the lymphocytes themselves (79).

5. Effect on lymphocyte proliferation

Certain plant mitogens induce blastogenic transformation of lymphocytes (43,50). Phytohemagglutinin (PHA) selectively activates T-cells (43,50,193) while Pokeweed mitogen (PWM) stimulates both T- and B-cells (50,193) and concanavalin A (con A) stimulates column-adherent T-cells (27).

Targowski (205) measured the *in vitro* incorporation of [$^{3}H$] thymidine into the DNA of pony lymphocytes cultured with PHA and found no significant

difference between control animals and those treated with a single 2 mg/Kg intravenous dose of DXM. Conflicting results were reported by Tarr and Olsen (206) who showed a 48% depression of PHA responses during a four day course of DXM treatment in adult horses. Con A stimulation was depressed 43% and PWM stimulation reduced 29% indicating preferential suppression of T-cell proliferation. Responses returned to normal within thirty-six hours after the last dose of steroid. These workers concluded that GCS administered in commonly used dosages could suppress cell-mediated immune responses of horses.

In contrast, no suppression of PHA responses was noted by Magnuson et al. (140) during a nine day course of DXM treatment (1 mg/Kg) of ponies. However, in vitro incubation of lymphocytes with prednisone inhibited PHA stimulation in a dose-dependent manner without inducing cell lysis. Lymphocytes from DXM treated ponies showed similar inhibition when prednisone was added to the culture medium indicating selection of GCS resistant T-lymphocytes does not occur during in vivo treatment. They advanced several possible explanations for these findings in the light of the more marked reduction of T-cell than B-cell numbers induced by DXM. Firstly, they suggested normally unresponsive B-cells may be responding to PHA under the stress of GCS treatment. Alternatively the effect could result from reduced numbers of circulating suppressor T-cells or thirdly, PHA-responsive lymphocytes may be less sensitive to GCS than other lymphocyte populations, a conclusion in agreement with that of Targowski (205).

Studies in calves conducted by Muscoplat et al. (158) showed marked depression of lymphocyte responses to PHA two to three days after initiation of a daily course of DXM administration (0.5 mg/Kg). However, PHA responses returned to normal during the treatment regimen leading these workers to conclude that a population of PHA responsive, steroid-resistant lymphocytes had been selected. This conclusion is in direct conflict with the above views expressed for the case in the horse (140).

In human subjects, Fauci and Dale (76) were unable to demonstrate any effect of in vivo doses of 100 mg or 400 mg of hydrocortisone on subsequent in vitro stimulation with PHA. In contrast, the response to Con A was

markedly suppressed as was PWM stimulation at the higher hydrocortisone dosage level. In a later study by Fauci (78) it was shown that hydrocortisone and prednisone caused only a slight depression of PHA responses, but this could be reversed by higher PHA concentrations. However, DXM caused a marked suppression of PHA responses at all concentrations of PHA used and also markedly reduced PHA-induced cellular cytotoxicity against labelled chicken erythrocytes. These findings were remarkable in that all three GCS agents caused an identical degree of lymphopenia. Whether the effects were due to the prolonged half life of DXM or a true qualitative difference from the other agents is not known (78).

Neither acute nor chronic hydrocortisone or cortisone acetate dosing of guinea pigs inhibits *in vitro* lymphocyte responses to PHA, PWM or Con A (11,77) despite a profound lymphopenia. It thus appears that in this species all functional lymphocyte subpopulations are equally depressed and their relative proportions are unchanged (11). In man, however, there is good evidence that GCS cause selective depletion of certain lymphocyte subpopulations (78,79,163).

This very confusing picture is made more so when *in vitro* responses to specific antigens are considered. In man (76) and in guinea pigs (11) GCS appear to inhibit antigen-induced blastogenesis more easily than that induced by mitogens. However, Targowski (205) showed no effect of *in vivo* DXM treatment of streptokinase-streptodornase sensitive ponies on the *in vitro* response of lymphocytes to the same antigen. Similarly, Magnuson et al. (140) showed chronic DXM treatment did not affect the *in vitro* response of lymphocytes from Keyhole Limpet Hemocyanin sensitive ponies to homologous antigen.

A possible explanation for this species disparity may lie in the fact that monocytes are thought to interact significantly in antigen-induced blastogenesis, at least in man (76,165). GCS induce a profound monocytopenia in man (76,78,168,228) but not the horse (140,167,205,206). Differential sensitivity of lymphocyte subpopulations to GCS administration, or selective sequestration of certain functional subpopulations from the circulation, may account for the finding that antigen-induced blastogenesis is

more easily suppressed than mitogen responses in man and guinea pigs (79).

Fauci et al. (79) concluded that much of the conflicting evidence on the effects of GCS on lymphocyte proliferation may be due to differences in culture methods and assay systems and that it is unlikely that direct suppression of lymphocyte proliferation is a major mechanism of action of glucocorticosteroids.

6. Effect of monocyte/macrophage and other CMI functions

Phagocytosis is an extremely important function of macrophages since it allows propagation of the immune response by early processing of antigens and later clearing of debris from areas of inflammation (230). Macrophages are also active in some forms of cell-mediated cytotoxicity (168) and are important effectors of delayed hypersensitivity (223,224). In addition to a marked circulating monocytopenia in man (76,78,168,228), GCS also cause depletion of macrophages at sites of induction of delayed hypersensitivity reactions (223). These effects are thought to be due to inhibition of promonocytes in the bone marrow, loss of mature monocytes from the peripheral circulation and diminished influx of monocytes from the peripheral circulation into areas of inflammation (208).

Chemotaxis of monocytes in response to serum, E. coli filtrate and lymphocyte chemotactic factor is inhibited by hydrocortisone in vitro (177). Glucocorticosteroids have been shown to block phagocytosis and reticuloendothelial clearance of opsonized and nonopsonized materials in vivo (7). Electron microscope studies have shown that this effect may be due to blocking of the earliest phase of phagocytosis, that is the adherence of foreign particles to the cell membrane (221). The effect of GCS on phagocytosis may, however, be biphasic; small doses being stimulatory (128) and large doses depressant (230).

The ability of macrophages to break down ingested material such as tubercle bacilli and heterologous erythrocytes was reduced in GCS-treated rabbits (128). In addition, GCS mediated depression of bactericidal and fungicidal activity of macrophages has been noted in in vitro studies (177). Whether this effect is mediated through the known stabilizing

effect of steroids on lysosomal membranes (54) or through intracellular metabolic or enzymic effects on the killing mechanism is not known.

From the above evidence, it has been suggested that GCS may suppress cellular immunity by directly interfering with the antigen-processing functions of macrophages (230), thus limiting access of antigen to immunologically competent cells (lymphocytes) (30). In addition, GCS may indirectly alter this process by limiting the access of macrophages themselves to sites where the antigen is initially deposited (79).

After activation by specific antigens or mitogens, T-lymphocytes secrete soluble products which perpetuate cellular immunity by recruitment of other cell types, especially macrophages. These soluble mediators include macrophage migration inhibitory factor (MIF), macrophage aggregating factor (MAF), monocyte chemotactic factor (MCF), skin reactive factor (SRF) and lymphotoxin (79). In vitro studies have shown GCS do not inhibit lymphocyte production of MIF (10), MAF (224) or SRF (169), but they do directly antagonize the effects of MIF and MAF on effector macrophages (79,223,224). These effects, coupled with those noted above, would explain the inhibition of delayed hypersensitivity skin reactions to tubercle bacilli seen in GCS treated guinea pigs in which a proportionally greater reduction of macrophage than lymphocyte numbers at the injection site is seen (223).

Direct cell-mediated cytotoxicity against particular target cells is a further function ascribed to certain T-lymphocytes (79). Cortisol has been shown to inhibit the cytotoxicity of mouse spleen cells for homologous target cells (179) and of rat lymphocytes for mouse target cells (46). It seems that GCS protect target cells from T-lymphocyte killer activity but do not alter the functional capacity of GCS-treated killer cells later tested without the drug (179).

The actions of glucocorticoids on isolated lymphocytes and on cell-mediated immune responses are summarized in Tables 1 and 2. In general, the available evidence presented above, suggests that the immunological processes dependent on T-lymphocytes are relatively resistant to the direct effects of GCS, but rather the functional alterations seen in the intact

Table 1. Effects of glucocortocosteroids on isolated lymphoid cells (Fauci et al. (79))

| Cell type | Recognized effects | Controversial effects |
|---|---|---|
| A. Monocytes and macrophages | 1. Decreased release from marrow | 1. Lysosomal stabilization |
| | 2. Decreased numbers in circulation | 2. Decreased responses to chemotactic factors |
| | 3. Decreased endocytosis and reticulo-endothelial system clearance | |
| | 4. Decreased bactericidal and fungicidal capacity | |
| B. Lymphocytes | 1. Decreased numbers in the circulation | 1. Lymphocytolysis and decreased viability |
| | 2. Altered migration patterns | 2. Altered cellular metabolism |

Table 2. Effects of glucocorticosteroids on cell-mediated immunologic processes (Fauci et al. (79))

| | Process | Recognized Effects | Controversial Effects |
|---|---|---|---|
| A. | Delayed hypersensitivity | Decreased expression of lymphocyte function | |
| B. | Antigen processing | Decreased lymphoid cell access to antigen in inflammatory sites | Macrophage processing per se |
| C. | Lymphocyte activation | Normal lymphocyte sensitization | |
| D. | Lymphocyte proliferation | Decreased antigen-induced blastogenesis | Mitogen blastogenesis |
| E. | Mediator production | Normal release of MIF[a], MAF[b] and SRF[e] | Release of LT,[c] MCTF[d] |
| F. | Response to mediators | Decreased effect of MIF , and MAF on macrophages | Effect on activity of LT and MCTF |
| G. | Cell-mediated cytotoxicity | Target cell protection | Killer lymphocyte function |

[a]Macrophage migration inhibitory factor.

[b]Macrophage aggregating factor.

[c]Lymphotoxin.

[d]Monocyte chemotactic factor.

[e]Skin reactive factor.

animal are compatible with drug-induced changes in lymphocyte circulation patterns. In contrast, several properties of macrophages are directly and indirectly affected including their circulation kinetics and access to sites of potential immunological reactions. Further, reduction in their ability to respond to soluble products released by activated lymphocytes results in marked suppression of cell-mediated immune responses (79).

7. Effect on B-cells and antibody production

The effects of GCS treatment on antibody production have not been fully elucidated. Many studies in man have failed to show any steroid induced suppression, perhaps due to the low doses used (230). However, Butler and Rossen (37) showed a significant reduction in Ig G concentrations in human volunteers thirty days and, in some cases, up to ninety days after a three to five day course of high dosage methylprednisolone therapy. Other antibody classes were also reduced in some patients. The plasma survival, plasma clearance and urinary excretion of Ig G were unaltered but there was a reduced entry of Ig G into the circulation as a result of inhibited antibody synthesis. High *in vitro* steroid concentrations have also been shown to reduce antibody production by isolated lymph node fragments from rabbits immunized against BSA and diphtheria toxoid (4).

Depression of *in vivo* antibody responses seems to depend upon when the GCS is administered in relation to antibody challenge. The hemagglutinin response of rabbits to SRBC is maximally suppressed by giving cortisone acetate twelve hours before antigen administration, an effect which may be due to inhibition of "helper" T-cells (63). Other studies have confirmed that the earliest phases of the antibody response are more vulnerable to the effects of GCS than the later phases and that the primary response is more easily inhibited than the anamnestic response (30,230). At least in the mouse, the steroid sensitive cell appears to be the peripheral unactivated B-lymphocyte (43) since if GCS are withheld until this B-cell, under the influnece of antigen, has matured into a plasma cell, the latter is found to be steroid resistant (150). It is

generally assumed that the ability of GCS to depress the humoral response lies in their destructive effects on lymphocytes and inhibition of protein synthesis and thus this effect is less marked in steroid resistant species (230).

Gabrielsen and Good (83) reported that, in addition to suppressing antibody synthesis, GCS can inhibit antibody mediated hypersensitivities such as local and systemic anaphylaxis and the Arthus reaction in actively or passively sensitized animals. Many variables affect these actions including the species of the animal, the agent used, the dose, route and time of administration and the amount of antibody present.

8. Effect on neutrophil and eosinophil function

The pronounced neutrophilic leukocytosis induced in many species by GCS is thought to be due to accelerated release of neutrophils from the mature bone marrow pool and their reduced egress from the blood to inflammatory sites (24). In addition, release of mature neutrophils from the marginated pool, which normally comprises more than 50% of the intravascular neutrophil population, may also play a part (205). The increased proportion of band forms seen during chronic GCS administration in the horse (140) may also indicate release of immature neutrophils from the bone marrow.

The markedly reduced accumulation of neutrophils at sites of inflammation is thought to be the major neutrophil deficiency induced by GCS and may, in part, be due to inhibition of their chemotaxis, a finding that has been quantitated by many methods in several species. A single dose of hydrocortisone reduced chemotactic accumulation of monocytes and neutrophils in a Rebuck skin window assay (24). In addition, GCS reduce granulocyte margination and adherence to vascular endothelium in response to inflammatory stimuli (69). Steroid induced inhibition of chemotaxis is currently thought to be due to alteration of the granulocyte surface rather than reduction of the host capacity to generate chemotactic factors (79).

Several studies have shown that GCS reduce phagocytic (49) and

bactericidal capabilities of neutrophils in vitro (49,142,177). Mandell et al. (142) showed a steroid-induced inhibition of NADH oxidase, an essential step in the intracellular production of hydrogen peroxide, which is a critical component of the normal oxidative killing mechanism. The GCS concentrations used in these in vitro studies is higher than can be achieved in vivo. Nevertheless, therapeutic doses of GCS have been shown to inhibit nitro blue tetrazolium dye reduction in human patients (42, 149), a defect similar to that seen in chronic granulomatous disease.

GCS have also been shown to reduce in vitro pyrogen production by leukocytes which may account for the steroid-induced suppression of fever seen in vivo (55).

The pronounced eosinopenia induced by GCS in a number of species is thought to result from redistribution of cells out of the circulating compartment (5). Archer (6) could find no evidence that hydrocortisone induced eosinophil lysis in the horse.

9. Effect on the complement system

The nine-component serum complement system plays a very important role in inflammatory processes. After activation by reaction of antibody with antigen, products are released by the complement cascade and are active as anaphylatoxins, chemotaxins for neutrophils, and opsonizing agents. Final assembly of the terminal components leads to cell lysis which further augments the inflammatory response. The activity of complement components on Hageman Factor can activate the kinin system, yet another mediator of inflammation (230).

Atkinson and Frank (7) showed a marked dose-dependent effect of cortisone acetate on the complement system of guinea pigs in vivo. Low dosage levels caused a significant elevation of whole complement activity, a striking rise in C1 and minimal increases in other components. Higher dosages led to a marked fall in overall activity and of all components except C1 and C9. Two weeks of therapy were required to give maximal effects and a similar period was needed for recovery of activity after dosing had ceased. This effect may be related to the fact that most

C' components are synthesized by macrophages which are known to be functionally inhibited by GCS, whereas C1 is produced by gut epithelial cells.

Gewurz et al. (86) showed hydrocortisone and prednisone inhibited immune hemolysis of SRBC by both guinea pig and human complement *in vitro*. This dose-dependent effect was thought to be due to inhibition of activity of all the major fluid phase components including C1 rather than a direct effect on the SRBC membrane. Other workers have indicated that hydrocortisone-induced inhibition of immune hemolysis may result from prevention of the attachment to the RBC surface of one or more complement component (110). That GCS inhibit this reaction by altering and stabilizing the target cell membrane has also been suggested (204).

10. Effect on interferon

Interferon is synthesized and released by leukocytes in response to certain viral infections and is thought to interfere with further infection by induction of synthesis of secondary new cellular proteins that prevent the replication of viral RNA (13). The effect of GCS on interferon is the subject of considerable debate. Kilbourne et al. (116) showed that cortisone inhibited the action of preformed interferon and its *in ovo* synthesis and release in chicken embryos inoculated with influenza virus. Fulton and Rosenquist (81) showed IBR virus to be a good inducer of interferon production by bovine tissues. Hydrocortisone decreased this production by bovine fetal spleen and peripheral blood leukocyte cultures but not alveolar macrophages.

In addition, Mendelson and Finland (146) have reported increased interferon production by mouse peritoneal leukocytes after large doses of GCS. Furthermore, Rosenquist (181) showed calves given doses of IBR virus and hydrocortisone had higher and more sustained interferon titers than those given IBR virus alone. This may be explained by the postulation of Smart and Kilbourne (195) that early inhibition of interferon synthesis by hydrocortisone allows additional viral replication and the increased amounts of virus stimulate further interferon synthesis.

## D. Equine Viral Rhinopneumonitis

### 1. Introduction

The disease syndromes caused by equine rhinopneumonitis virus (ERV) have been given considerable attention since the condition was first described in 1933 (56). At that time, Dimock and Edwards (56) recognized the contagious nature of equine abortion and suggested that a filterable virus, equine abortion virus, was responsible. Manninger and Csontos (143) found that mares inoculated with equine abortion virus developed a transient respiratory syndrome similar to equine influenza and they thus proposed that virus abortions were a manifestation of infection of pregnant mares by the influenza virus. Similar conclusions were recorded by Kress (122,123) who observed bronchopneumonia among horses in contact with aborting mares and involvement of the respiratory tract of aborted fetuses. Hansen and Holst (92) reported the occurrence of virus abortions which were preceded in the same herd by an outbreak of respiratory disease with clinical manifestations of equine influenza. The studies of Doll et al. (59) confirmed that equine abortion virus was indeed capable of producing an influenza-like syndrome in experimentally inoculated horses, but this agent was distinct from equine influenza virus.

Equine abortion virus, now known as equine rhinopneumonitis virus (ERV) and equine herpes virus 1, is currently recognized as a major cause of disease in horses (14,170) with a high incidence throughout the world (14,26,44,66,161). Abortion in mares and interruption of training and performance caused by the respiratory form of the disease contribute to considerable economic loss (14,170). Recently, ERV infection has been associated with two further disease syndromes; namely the birth of weak, nonviable foals (14,36,57,66,170) and a paretic condition in older horses (40,102,103,131,132,200).

## 2. Etiology and pathogenesis

All isolates of ERV are considered to be antigenically identical (14), although it has been suggested that two biological subtypes may exist (26). This view is based on the finding that, although the respiratory form of the disease is ubiquitous, abortion and other manifestations of infection are rarely encountered in some countries (26).

The virus is infective by contact exposure or by inoculation via the nasal, oral, tracheal, conjunctival or parenteral routes (102). The work of Bryans (35) has shed considerable light on the pathogenesis of ERV infection. Natural exposure and inhalation of the virus is followed by invasion of the superficial tissues of the nasopharynx with rapid viral proliferation. A barrier to infection at that site is thought to be a reflection of the serum virus neutralizing antibody titer which, if high enough, can block viral penetration. Lower levels of antibody permit proportionately limited infection. Local tissue destruction resulting from viral proliferation stimulates infiltration of phagocytic cells which ingest and become infected by the virus. Passage of these phagocytes into the circulation results in a viremia associated with mononuclear cells of the buffy coat (65). The virus is thus seeded to distant organs, including the pregnant uterus and central nervous system (CNS), being protected from circulating antibody by virtue of its intracellular location (35). Direct cell to cell viral spread (35) allows invasion of the fetus where maternal antibodies are absent due to the placental barrier (14,65).

A similar mechanism for viral infection of the CNS has been postulated in light of the finding that endothelial cells of vessels are the first to become infected and from which centrifugal infection of the parenchyma occurs (103).

## 3. Epidemiology

ERV occurs most frequently in areas of concentrated horse breeding and at locations of transient contact such as shows or sales (14). The latter allows spread to other farms in different locations. That

infection is widespread was revealed by a recent survey which indicated that eighty-three percent of Australian horses had been exposed (9). On many farms, in certain geographical locations, infection is enzootic and outbreaks occur annually in the fall and winter (14). At this time, the foals' colostral antibody titers have waned to unprotective levels and because they are grouped together at weaning, the risk of infection is increased. Eighty percent of young horses (mainly weanlings or those experiencing their first exposure) on some unprotected farms will show respiratory symptoms at this time (60) and eighty-one to one hundred percent will show seroconversion indicating infection (59).

Infected weanlings appear to be the principal reservoir of infection for in-contact adult horses (14,59) which generally do not show marked evidence of disease due to the immunizing influence of multiple prior infections (59). However, infection of pregnant mares may, depending on their immune status, result in viral invasion of the fetus which is not protected by maternal antibody. After an incubation period of three to twelve weeks, the infected fetus is aborted, usually in the latter stages of pregnancy (14), and a further focus for viral dissemination is created.

4. Clinical disease syndromes

a. Respiratory disease In 1954, Doll et al. (59) provided an excellent documentation of the clinical signs produced by intranasal and intravenous inoculation of weanling horses with virulent ERV. The experimental and naturally-occurring disease syndromes have since been further described (14,26,170).

After an incubation period of two to ten days (14) or even up to twenty days (26), young horses which have not been previously exposed develop serous nasal and ocular discharges with congestion of the conjunctival and nasal mucosae. A fever, which is often biphasic with higher afternoon temperatures, develops and may range up to 105°F; it can persist for one to seven days. General depression and loss of appetite may occur but are usually of limited degree in horses with uncomplicated

infections, especially if they are rested. Slight enlargement of the mandibular lymph nodes and edema of the distal limbs may be present. A leukopenia, persisting for up to thirteen days after experimental inoculation, is also observed in natural cases and is due to a marked depression of neutrophil counts, other blood elements remaining relatively stable (59).

In the uncomplicated disease, recovery is complete within one to two weeks but complications such as mucopurulent rhinitis, pharyngitis and coughing are common and generally due to secondary bacterial invasion. Pneumonia is rare but can develop in young foals (26). Animals rarely die as a result of ERV infections and, indeed, inapparent infections are common especially in older, previously exposed horses (26).

Pathologic lesions in uncomplicated cases are confined to the upper respiratory mucosa which exhibits edema, congestion, and petechial hemorrhages (14).

b. Neurologic disease In 1966, Saxegaard (187) reported a neurologic syndrome occurring during an outbreak of ERV abortion in horses in Norway. This condition has since been recorded in association with natural cases of respiratory disease and abortion (40,131,200) and following inoculation of virulent (102,103) and vaccinal virus (132).

Sprinkle (200) observed six cases in a single stable beginning ten days after an adjacent mare aborted a nine month fetus. The animals, mainly geldings, at no time showed evidence of depression, anorexia or respiratory disease but all had fevers up to 106.2°F one to twelve days prior to the development of neurological signs. One horse had complete hemiparesis and another was unable to defecate. The predominant signs, however, were inability to urinate and posterior ataxia which progressed to total posterior paralysis and recumbency necessitating euthanasia in one horse. Most of the cases recovered over a period ranging from a few hours to eight months. The same author reported transient signs of posterior weakness and loss of bladder control in another group of horses which showed concomitant signs of respiratory disease. Many in-contact

horses demonstrated transient posterior ataxia during the outbreak (200).

Other signs observed during the course of this disease include lethargy, and edema of the hind limbs (40), stiffness and reluctance to move (102), urinary incontinence (131,132), sensory nerve deficits (132), and total paralysis, except for the head, without loss of pain sensation or anal reflexes (131). Elevated protein levels in the CSF have been consistently observed in some investigations (102,103,132).

Experimental studies using the Army-183 strain of virus showed that a high percentage of pregnant mares developed neurological signs six to eight days after subcutaneous inoculation, but this was related to the stage of gestation. Nonpregnant mares did not develop signs, whereas all the mares which were three to nine months pregnant did. Mares which were ten months in foal at the time of inoculation showed no neurological signs but some aborted (103). Neurological and abortigenic symptoms can occur together in a single mare but generally do not. This may be related to the differences in the incubation periods of the virus in the two syndromes or may reflect variations in the host response (40).

Pathologic lesions reflect a nonsuppurative disseminated meningoencephalomyelitis involving the brain and spinal cord and are well-described (40,102,103,131,132). Necrotizing vasculitis, being most prominent along the meningeal surface of the brain and cord (103), gives rise to focal areas of malacia (40,132). Axonal swelling and perivascular mononuclear cuffs are consistent lesions and the grey and white matter are equally affected (102,103). Trigeminal ganglioneuritis was evident in many cases and peripheral neuritis was seen in three horses with unilateral lameness in one study (131). The inciting vasculitis generally involves the smaller arteries and veins (103,132).

ERV has been isolated from nervous tissues of some clinical cases (40,131,187), but this is not easily accomplished. Similarly, typical intranuclear inclusion bodies have not been reported in the CNS of the affected animals (40). Jackson and Kendrick (102) found severely affected mares all had preinfection neutralizing antibody titers and showed a more rapid titer rise than less severely affected mares. The above

findings, taken together, led to the suggestion that an immunological reaction may be involved in the production of vascular lesions (102). Evidence for direct viral invasion of the neural blood vessels is, however, equally strong (103).

c. Abortion The pathogenesis of this syndrome is not well-understood even though ERV has been shown to be the most common cause of equine abortion in some areas (170). Abortigenic infection can be induced in mares with high virus neutralizing antibody titers, but conversely low antibody titers are not necessarily synonymous with susceptibility (35). ERV-induced abortion can occur in the same mare in successive years indicating immunity is short-lived (59).

Infection of pregnant mares is generally not accompanied by respiratory symptoms of the disease (14,26,59), but rather an incubation period of three to sixteen weeks (26) is followed by spontaneous abortion occurring without warning (14,26,59). The mares may or may not have mammary development before going into labor, the fresh fetus is rapidly expelled, and the placenta soon follows. Future breeding performance is generally unimpaired (14). Most aborting mares do so in the last third of pregnancy, particularly in the eight to ten month period, but some abortions do occur as early as the fifth month (26).

Jackson et al. (103) found vasculitis in the vessels of the endometrium to be a consistent lesion in experimentally infected mares. These workers also observed placental lesions consisting of congestion and hemorrhage in the chorio-allantois. Local edema of the feto-maternal junction with separation of the chorio-allantois from the endometrium is also commonly encountered (26).

Pathologic lesions in aborted fetuses have been well-documented (14,26,44,103,170,222). Gross changes include icteric discoloration of the foot pads, intense inflammation of the turbinates, excessive fluid in the pleural and peritoneal cavities, edema and atalectasis of the lungs, and subcutaneous edema. Hemorrhages are often seen on the upper respiratory mucosa and pericardium, in the oral cavity and digestive

tract and other organs throughout the body. Focal necrosis of the liver is a prominent feature, but similar lesions occur in the lung, spleen, lymph nodes, thymus and other organs.

Histological examination reveals eosinophilic intranuclear inclusion bodies in hepatic cells and in the alveolar and bronchiolar epithelium. Viral antigen in these and other locations is noted by immunofluorescence staining and virus isolation. Focal necrosis in many organs is the basic pathologic process associated with ERV infection, but vascular compromise, as indicated by widespread exudation of plasma protein, is also prominent (103). Viral inclusions have been found in all blood vessel components including the adventitial cells (222).

ERV-infected fetuses may lack gross lesions, but microscopic and immunofluorescence examinations will usually confirm the diagnosis (44).

d. Early neonatal death syndrome There are a number of reports of disease during the early neonatal period related to the in utero infection of the fetus late in gestation (14,26,36,57,170). Typically, foals are born at or near full-term but are weak, unable to stand, and exhibit severe respiratory distress and susceptibility to secondary infection. Death may occur shortly after birth or three to four days later (14).

Dixon et al. (57) reported histopathological and virological evidence that twenty-nine of forty-three foal deaths, on a single Australian farm which foaled one hundred and forty-five mares, were attributable to ERV infection. Some of the foals were stillborn or died very quickly, while others were weak, depressed and unable to stand or nurse and died within twenty-four hours. Some had raised rectal temperatures, and all had markedly elevated heart and respiratory rates with increased bronchial sounds and sibilant rales on auscultation. A third group of foals were essentially normal from birth until eighteen to twenty-four hours of age, at which time they developed severe respiratory distress along with signs similar to the preceding group and died by twenty-four to seventy-two hours despite intensive treatment. All foals showed a severe leukopenia and neutropenia and most were hypogammaglobulinemic. An absolute lymphopenia

was seen in some foals.

The major pathologic lesions included acute focal necrotizing bronchiolitis, pulmonary edema with accumulation of fluid and froth in the airways, and hydrothorax. Liver lesions, as seen in aborted fetuses, were not a prominent feature. Petechiae were found in a number of organs including the heart, adrenals, spleen and mesenteric lymph nodes which were also frequently enlarged and edematous. Eosinophilic intranuclear inclusions could be demonstrated in bronchiolar epithelial cells and ERV was isolated from the lungs and sometimes other organs. In addition, one-third of the foals showed evidence of antemortem septicemia.

Bryans (36) recently reported on a series of nine foals which presented clinical signs similar to those in the above investigation. However, the majority were normal at birth, becoming ill during the first week of life. Diarrhea and intensely reddened conjuctival and buccal membranes were prominent abnormalities in addition to those thus far described. Hypogammaglobulinemia was seen in those foals tested but, in contrast to the hemogram findings of Dixon et al. (57), lymphopenia was a marked feature with a neutrophilic leukocytosis developing during the course of the disease which lasted from a few days up to two weeks before death. All foals showed pathologic changes referable to secondary infection with a variety of bacteria. Other universal lesions included interstitial pneumonia, adrenal hyperplasia with focal cortical hemorrhages, hypoplasia of the thymus and spleen with marked depletion of splenic lymphocytes, and massive necrosis of all elements of the thymus. Viral inclusions were not seen in any of the tissues examined but ERV was isolated from three of the foals.

These workers provided histological evidence that the spleen of the fetal and neonatal foal contains the majority of the total lymphocyte population. They thus concluded that destruction of this compartment, as well as the thymus, by ERV severely compromises the foal's ability to combat bacterial infections. The ability of the animal to recover from the disease would depend upon its escape from unresolvable thymic damage and its ummunological compensation for damage to the spleen. These

workers further speculated that a fatal case of immunodeficiency in a yearling thoroughbred colt (136) was related to intrauterine ERV infection with subsequent inability to reconstitute its immune capability.

5. The immune response to ERV

Extensive investigation of the immune responses of horses to virulent and vaccinal ERV have shown them to be complex and no clear understanding has been gained (14,35,59,65,66,85,102,161,170,209,226,227).

Doll et al. (59) intranasally inoculated 6-9 month old weanlings with virulent virus and demonstrated the development of complement fixing (CF) antibodies by the eleventh or twelfth day. Titers peaked, at between 1:64 and 1:256, fourteen to twenty days postinoculation and remained elevated at sixty days. In studies employing a modified live virus (MLV) vaccine,[1] Bass et al. (14) showed that young ponies with prevaccinal serum neutralizing (SN) antibody titers of less than 1:4 developed an eight to sixteen-fold titer rise by four weeks after a single intramuscular dose. A rapid further rise was seen after virulent challenge, titers being higher than those of nonvaccinated control ponies. In addition, booster vaccination of unchallenged ponies forty days after the primary dose resulted in a further three-fold SN titer rise. Similarly, pregnant mares with mean antibody titers of 1:12 developed a four-fold rise following a two dose vaccination series. An investigation by Piersen (170) using the same vaccine demonstrated a significant (three-fold) titer rise in yearlings after primary vaccination but no anamnestic response in samples taken eight weeks after a booster dose.

SN antibodies generally begin to appear seven to ten days after infection in normal horses (227) but the situation seems somewhat different in gnotobiotes which did not demonstrate an appreciable response until three weeks postinfection in the studies of Thomson and Mumford (209). Peak titers were not attained until the fifth to seventh week, whereas conventional foals displayed a peak at two-to-three weeks with lower overall antibody levels. Thus gnotobiotes seem to display a delayed and prolonged humoral response to infection (209).

[1] Rhinomune®, Norden Laboratories Inc., Lincoln, Nebraska.

Absorption of specific colostral antibodies by nursing foals furnishes them with anti-ERV titers equivalent to those of their dams by twenty-four hours after birth. Levels are decreased, but still significant, in two month old foals but have waned to low levels by three-to-four months of age (66,161).

Furthermore, the ability of horses to mount endogenous humoral responses varies greatly depending on their age when vaccinated or challenged (66,85,161). Dutta and Shipley (66) found a negligible SN antibody response in one-to-four month old foals to either primary or booster doses of MLV vaccine,[1] irrespective of the preexisting antibody titer or exact age of the foal. A similar effect was observed by Neely and Hawkins (161) in two-to-seven month old foals subjected to either vaccination or natural challenge. In contrast, mares in their study developed a very rapid, one-to-five-fold titer increase indicative of an anamnestic response. In addition, Gerber et al. (85) reported that, whereas pregnant mares and eighteen-to-twenty-one month old horses developed a four-to-sixty-four-fold titer increase beginning one week after vaccination, six-to-eight month old horses showed little or no serological response to either the primary or booster dose. These effects may indicate a lack of maturity in the humoral immune system of the young horse but more likely reflect their lack of previous exposure to the virus (85,161).

Preexisting SN antibody titers also appear to affect the response to vaccination or challenge (35,161). Neely and Hawkins (161) observed diminished serological responses in horses of all ages with prevaccinal titers of more than 1:64; titers of this magnitude being protective against both respiratory and abortigenic infection. Similarly, Bryans (35) found that animals with SN titers of more than 1:100 failed to develop anamnestic antibody responses, viremia, or clinical infection when challenged. In addition, titers were generally higher in those animals from which the virus could not be isolated than in those having a viremia. Bryans thus concluded that resistance to infection is a reflection of the serum neutralizing antibody titer and is mediated via the

[1] Rhinomune$^{R}$, Norden Laboratories Inc., Lincoln, Nebraska.

barrier it creates on or in the mucosa of the nasopharynx and related structures (35).

Resistance to the respiratory form of ERV infection is, however, short-lived in young horses (14), being in the order of four-to-six months after vaccination (66,161) and probably of similar duration following natural challenge (14,35). In older horses, antibodies are regularly formed and persist longer than in foals due to the additive effects of repeated exposure (35). However, titers are transient, even after natural exposure in pregnant mares, and without reexposure will fall appreciably within four months of infection (161).

In spite of the protective role ascribed to humoral mechanisms, it has become increasingly obvious in recent years that resistance to ERV infection is not adequately explained by the production of SN antibodies alone (85,226,227). Mares have aborted in the presence of high tiers (35,66) and similar titers have been seen in normal foaling and aborting mares (66). Animals which develop the paralytic syndrome can have significant titers which become markedly elevated following infection (102). Furthermore, viremia can occur in the presence of neutralizing antibody (35) but, in contrast, foals develop a poor humoral response to ERV but can still remain free of symptoms for up to six months (66,161). These findings have led to the suggestion that cell-mediated immune (CMI) mechanisms may be involved in resistance to this disease (65,66,85,209, 226,227). Wilks and Coggins (226) noted that CMI plays a major role in recovery from those viral infections in which plasma viremia is not important and infected cells bear virus-specific antigens on their surface, as is the case with ERV.

In vitro lymphocyte blastogenesis in response to PHA (65,209,226) or ERV antigen (65,85,209,226) and in vitro cytotoxicity assays (227), have been used to quantitate CMI responses following sensitization with ERV. Live ERV has, however, been shown to suppress the proliferative response of nonimmune lymphocytes to both PHA (209) and ERV (85), but not the response of immune lymphocytes from vaccinated horses (85). This may reflect an inhibition by live virus of the metabolic processes

involved in $^{3}$H-thymidine uptake (209). Gerber et al. (85) reported equivalent stimulation induced in immune lymphocytes by both live and heat-inactivated ERV antigen whereas Thompson and Mumford (209) showed heat and u.v.-inactivated virus gave much better responses than live virus. These discrepancies may reflect the different culture methods used, but most investigators now use inactivated virus (65,226). Autologous serum appears to inhibit responses to viral antigen, but not to PHA, in some systems (226).

Lymphocytes from two week old foals demonstrated appreciable PHA-induced stimulation indicating that foals are competent with regard to their CMI responses from a young age (209). However, individual horses tend to show marked variability in PHA responses making interpretation of results difficult (226).

The development of lymphocyte sensitivity following vaccination or infection seems to be negatively correlated with the appearance of SN antibody. Wilks and Coggins (226) showed lymphocyte sensitivity developed as early as two days and peaked at seven-to-fifteen days following inoculation of ponies with virulent virus. The decline in responsiveness after this peak was associated with increasing SN antibody titers. Gnotobiotes became sensitized more slowly with responsiveness appearing one-to-two weeks postinoculation (209). A peak at four weeks was followed by a decline as SN titers became maximal and as these waned a second peak of responsiveness appeared at eight weeks and was still significant ten weeks postinoculation. Gnotobiotic foals inoculated with attenuated virus developed poorer lymphocyte sensitization than those given virulent virus and showed no anamnestic CMI response when given a booster dose, at which time SN titers became elevated (209). Conventional foals in this study showed a markedly different response, attaining a single peak of sensitization, much lower than that of gnotobiotes, two-to-three weeks postinoculation.

In contrast to their poor humoral responses, young (six-to-eight month old) horses developed markedly increased CMI responses following administration of a MLV vaccine[1] in the studies of Gerber et al. (85).

[1] Rhinomune$^{R}$, Norden Laboratories Inc., Lincoln, Nebraska.

Stimulation indices peaked thirteen weeks postvaccination and were still evident at twenty-one weeks. Eighteen-to-twenty-one month old horses developed a rapid humoral response but lacked lymphocyte responsiveness until eight weeks after a booster vaccination, at which time SN titers fell precipitously. These workers suggested that the mechanism of antibody production may be less well-developed than that of the cellular response in young horses. They also hypothesized that the apparent inhibition of CMI responses by antibody may be due to blockage of the antigenic determinants on T-cells by antibody. These two resistance mechanisms may be regulated by the cellular interaction of amplifying and suppressor T-cells, B-cells, and free as well as cell-associated antigen (85).

The exact duration of the CMI response to ERV infection is not known. Wilks and Coggins (226) suggested that the lack of blastogenic response of lymphocytes from four ponies having serological evidence of prior infection may have been due to the more rapid waning of cellular than humoral immunity. Similarly, the susceptibility of horses with SN titers to infection may reflect their inability to mount an initial CMI response or the waning of induced CMI responses to below protective levels.

Gerber et al. (85) have demonstrated significant suppression of blastogenic responses to ERV during pregnancy (especially the latter stages), but humoral responses remained unimpaired as indicated by a sixteen-fold increase in SN titers following virulent challenge late in gestation. The steroid environment of pregnancy or lymphocyte turnover may have been responsible for this effect which could allow the dissemination of virus and abortion, in the presence of antibodies, late in gestation. However, Dutta and Campbell (65), were unable to find significant differences in stimulation or any correlation between the antibody titers and stimulation indices in any of the mares studied.

The possibility of a role for cytotoxic reactions in recovery from ERV infection was recently introduced by Wilks and Coggins (227). They showed that in vitro cytotoxicity of leukocytes and serum, alone or in combination, for labeled ERV-infected cells appeared as early as one day postinfection. These effects peaked at five days, returned to normal between

seven and ten days as SN antibody appeared, and showed a second peak at fifteen days. Lysis of virus-infected cells by this mechanism could expose this cell-associated virus to the neutralizing effects of antibody *in vivo*. However, viral antigens are late to appear on the cell surface so that cells capable of inducing lysis would not be able to recognize infected cells until late in the infection. In addition, neutralizing antibodies do not appear until at least seven days postinfection, thus early release of virus would not be in the best interests of the host and could even aid in viral spread (227).

The relative significance of the various cellular and humoral mechanisms in recovery and future protection of horses against ERV infection remains unclear. That both responses do occur is now well-established and it is likely that they act in concert, to a greater or lesser extent, in different situations.

# III. MATERIALS AND METHODS

## A. Experimental Animals

Twenty-one pregnant pony mares, comprising twenty Shetland and Shetland cross and one Welsh type, were maintained on pasture and observed twice daily for signs of impending parturition. The mares had been pasture bred by Shetland stallions the previous spring and summer, and had been individually identified by freeze branding during a previous study. Their foals were given the same number as the mare plus the suffix "77" to indicate their dam and year of birth (1977). Soon after foaling, the mares and foals were individually housed indoors in pens approximately ten feet by ten feet and fed twice daily a diet of hay and corn with water being available ad libitum. The birth weight, markings and sex of the foals was noted and they were included in the study at between twelve and twenty-four hours of age (day 1) depending on their time of birth. At this time the foals were randomly assigned to one of four experimental groups. The major portion of the study concerned investigation of the effects of dexamethasone and rhinopneumonitis virus on immune responses and cortisol levels in these foals and thus group assignments were as follows:

Group 1 - Control - no dexamethasone, no ERV
Group 2 - Virus control - dexamethasone, no ERV
Group 3 - Experimental - dexamethasone and ERV
Group 4 - Dexamethasone control - no dexamethasone, ERV

During the first twelve days of investigation, the ERV-inoculated groups were individually housed in a separate area from the noninoculated groups. While the noninoculated groups remained in individual pens until the twenty second study day, the ERV-inoculated groups were communally housed in a large shed after the twelfth day. Foals in all four groups were turned out into a large wooded pasture and allowed to mix freely when they reached twenty-two days of age. Sampling during this period was performed in a corral in one corner of the pasture.

The remaining part of the study involved comparison of the plasma

cortisol concentrations of adult ponies and horses with those of the young foals. Group 5 consisted of five Shetland ponies in a group of about ten, owned by the Department of Veterinary Clinical Sciences, Iowa State University, which were sampled prior to their use in the surgical laboratory. These ponies had been purchased at local sales and housed together in a large pen for at least ten days prior to the start of the study. They were maintained on a ration of hay with a small amount of grain fed twice daily.

Group 6 comprised eight nongravid adult mares of the quarter horse (5), Arabian (2), and Thoroughbred (1) breeds and owned by the Department of Veterinary Clinical Sciences, Iowa State University. These mares were housed in individual tie stalls and fed hay and a grain mixture twice daily during the study.

## B. Immunizing Agents Used

### 1. Equine rhinopneumonitis virus

The virulent rhinopneumonitis virus used in the study was the Army challenge strain #183 with a titer of approximately $10^7$ infective particles per ml. This virus was kindly donated by Norden Laboratories, Inc., Lincoln, Nebraska.

### 2. Modified live virus

Modified live rhinopneumonitis virus was prepared just prior to use by aseptic rehydration of a commercially available lyophilized equine cell line origin modified live virus[1] with 2 ml of the sterile diluent supplied with the preparation. This dilution produced a release titer of $10^6$/ml. For booster vaccination, 2 ml of this vaccine was aseptically injected intramuscularly according to the manufacturer's directions. In initial challenge studies, either 6 ml or 10 ml of this preparation was aseptically injected intravenously into the jugular vein using a 1" 18G needle.

---

[a] Rhinomune$^R$, Norden Laboratories, Inc., Lincoln, Nebraska.

3. Brucella antigen

Brucella antigen was produced from a commercial preparation of lyophilized live Brucella abortus strain 19 cells[1] reconstituted to 25 ml with the sterile diluent supplied, giving an average concentration of $2x10^{10}$ organisms per 1 ml (range $1-3x10^{10}$). The bacteria were killed by heating in a water bath at 65°C for one hour. This inactivated bacterin was then stored in capped vials at 4°C until used.

4. Sheep red blood cell antigen

Sheep red blood cell antigen was prepared fresh before use from a stock suspension of sheep blood which had been collected aseptically into an equal volume of sterile Alsever's solution. Approximately 15 ml of this suspension was then aseptically transferred to sterile 16x125 mm screw capped glass tubes and centrifuged at 350xg for 10 minutes. After aspiration of the supernatant plasma, the cells were resuspended in sterile normal saline solution (NSS), by gentle rotation of the tube, and centrifuged as above. The supernatant was aspirated and the cells washed three times in NSS, followed by addition of NSS to make a 10% suspension.

## C. Experimental Protocol

1. Foals

A printed sampling protocol sheet was prepared for each group and used to record sampling information separately for each foal. Group assignments are shown in Table 3. The foals in all four groups were

Table 3. Group assignments

| Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|
| EB177 | 977 | 677 | 3477 |
| 477 | 1377 | 877 | 5877 |
| TGC77 | 2977 | 577 | 377 |
| JP77 | 1177 | 5777 | 1777 |
| | 1977 | 277 | 2377 |
| | DD77 | 3077 | |

[1]Brucella Abortus Vaccine, Jensen-Salsbery Laboratories, Kansas City, Missouri.

subjected to the treatment and sampling procedures which follow:

(i) Brucella antigen was administered on day three by subcutaneous injection in the neck using a 1", 20G needle.

(ii) Lymph node biopsies were collected from the right submandibular lymph node between days twelve and nineteen, but in most cases on day thirteen or fourteen.

Anesthesia was induced by intravenous administration of xylazine[1] at the dose of .5 mg per pound followed in five minutes by intravenous ketamine hydrochloride[2] at the rate of .8 mg per pound. Foals were positioned in dorsal recumbency and the area over the right submandibular lymph node was clipped and prepared by two washes with a povidone-iodine scrub[3] followed by the application of alcohol. A 2 cm skin incision was made directly over the lymph node which was then exposed by blunt dissection of surrounding tissues. A portion of the node was then resected and placed in 10% normal balanced formalin in labeled jars for fixation prior to histological examination. Bleeding was usually minimal during this procedure, but, if necessary, vessels were ligated using 00 chromic catgut.[4] The subcutaneous tissues were closed using a simple continuous suture of 00 chromic catgut,[4] and the skin was closed using a continuous interlocking suture of 0 monofilament nylon.[5] The whole procedure usually lasted ten to twelve minutes and by fifteen to twenty minutes after anesthetic induction, the foals were standing and could be returned to their stalls.

(iii) Blood samples for hemotological examination were collected on days one, four, seven, twelve, and twenty-two, by jugular venipuncture with a 1", 20G needle, blood being drawn into 7 ml evacuated

---

[1] Rompun®, Haver-Lockhart, BayVet Division, Cuttler Laboratories, Inc., Shawnee, Kansas.

[2] Ketaset®, Bristol Laboratories, Bristol-Myers Co., Syracuse, New York.

[3] Betadine Surgical Scrub, The Purdue Frederick Co., Yonkers, New York.

[4] Ethicon®, Ethicon, Inc., Somerville, New Jersey.

[5] Ethilon®, Ethicon, Inc., Somerville, New Jersey.

tubes containing EDTA as an anticoagulant[1]. These samples were submitted to the Clinical Pathology Section, Department of Pathology, College of Veterinary Medicine, Iowa State University for evaluation of hemoglobin concentration, packed cell volume (PCV), total white blood cell count and plasma protein and fibrinogen concentrations. Blood smears were prepared and stained with Wright-Giemsa Stain[2] and stored dry for later evaluation of white and red blood cell morphology and differential white blood cell counts. All slides were examined by the investigator under the oil-immersion objective of a microscope. Two hundred leukocytes were counted to determine differential counts, from which absolute leukocyte counts were calculated.

(iv) Blood samples for anti-ERV antibody titer evaluation were collected into evacuated tubes,[1] as described above, on days one, four, twelve, twenty-two, thirty-five and on the day of ERV vaccination (day forty). Further samples were collected ten and seventeen days later (days fifty and fifty-seven, respectively) to assess the serologic response to vaccination. These samples were maintained at room temperature for about four hours before centrifugation at 1000xg for ten minutes. The serum was harvested and stored in 12x75 mm capped plastic tubes[3] at -20°C. Rhinopneumonitis titers were determined by Norden Laboratories Inc., Lincoln, Nebraska, employing a serum-neutralization procedure in a microtiter system using the constant virus, varying serum method. Serum samples from the dams, collected prior to foaling or soon thereafter, were also evaluated for ERV titers.

(v) Plasma samples for cortisol determination were collected twice daily at between 7:45 am and 8:30 am and again between 5 pm and 5:30 pm on days one through twelve and on days seventeen and twenty-two. Blood was collected by direct jugular venipuncture under minimal restraint

---

[1] B-D Vacutainer, Becton, Dickinson and Co., Rutherford, New Jersey.

[2] Wright-Giemsa Stain, Diagnostic Division, Fisher Scientific Co., Orangeburg, New York.

[3] 2058 tube, Falcon[tm] Division, Becton, Dickinson and Co., Rutherford, New Jersey.

into 7 ml evacuated EDTA tubes.[1] Every attempt was made to minimally excite and stress the foals and in all instances sample collection was accomplished within one to two minutes of initially approaching the animal. Samples were centrifuged at 1000xg for five minutes within thirty minutes of collection, and the plasma was then harvested into 12x75 mm capped plastic tubes[2] and immediately frozen and stored at -20°C.

(vi) Samples for anti-brucella antibody titer determination were collected on the morning of days one, three, four, twelve, seventeen, and twenty-two. Some samples were of plasma and others of serum, collected as described above and stored at -20°C. Samples from the dam collected prior to or shortly after foaling were also evaluated for brucella titer.

(vii) 2 ml of 10% sheep red blood cell antigen (SRBC) was administered intravenously using a 1" 20G needle at between day thirteen and seventeen, usually day fourteen.

(viii) Serum samples were collected as previously described on days twelve, twenty-two and thirty-five and stored at -20°C for anti-SRBC antibody titer determination.

(ix) 35 ml of blood was collected for harvesting of lymphocytes for in vitro studies on days four, twelve and seventeen. After aseptic jugular venipuncture using a 16G 1½" needle, blood was aspirated into a sterile 125 ml Erlenmeyer flask using a sterile suction device. Several applicator sticks had been attached together in the shape of a cone in the middle of the Erlenmeyer flasks prior to sterilization. These sticks facilitated defibrination of the blood by gentle swirling of the flasks during and immediately after blood collection.

(x) Throughout the course of the investigation up to day twenty-two, the foals' rectal temperatures were measured and recorded along with any abnormal clinical signs.

(xi) Superimposed upon the above sampling and treatment regimen,

---

[1] B-D Vacutainer, Becton, Dickinson and Co., Rutherford, New Jersey.

[2] Azium[R], Schering Corporation, Kenilworth, New Jersey.

foals in groups 2 and 3 received daily intramuscular injections of 4 mg (2 cc) of a commercial dexamethasone (DXM) preparation[1] from day one to day six. The dose was then reduced over the next three days so that the foals received 3 mg of DXM on day seven, 2 mg on day eight, and one mg on day nine.

(xii) On day four, foals in groups 3 and 4 received intravenous doses of either 5 ml of the virulent ERV preparation or either 6 ml or 10 ml of the modified live ERV preparation. Use of modified live virus was necessitated by the death of two foals following administration of the virulent virus. Table 4 summarizes the virus challenges administered.

Table 4. Virus challenges administered

| Group 3 | | Group 4 | |
|---|---|---|---|
| Foal # | Treatment | Foal # | Treatment |
| 677 | MLx5[a] | 3477 | MLx3 |
| 877 | MLx3[b] | 5877 | MLx3 |
| 577 | MLx5 | 377 | V[c] |
| 5777 | V | 1777 | MLx5 |
| 277 | MLx3 | 2377 | V |
| 3077 | V | | |

[a] MLx5 = 10 ml Rhinomune$^{R}$.

[b] MLx3 = 6 ml Rhinomune$^{R}$.

[c] V = virulent ERV.

Table 5 summarizes the experimental protocol used in this investigation.

[1] Azium$^{R}$, Schering Corporation, Kenilworth, New Jersey.

Table 5. Experimental protocol[a]

| Day | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|
| 1 | A,C,E,H,R | A,C,D4,E,H,R | A,C,D4,E,H,R | A,C,E,H,R |
| 2 | C,R | C,D4,R | C,D4,R | C,R |
| 3 | B,C,E,R | B,C,D4,E,R | B,C,D4,E,R | B,C,E,R |
| 4 | A,C,E,H,I,R | A,C,D4,E,H,I,R | A,C,D4,E,H,I,R,V | A,C,E,H,I,R,V |
| 5 | C,R | C,D4,R | C,D4,R | C,R |
| 6 | C,R | C,D4,R | C,D4,R | C,R |
| 7 | C,H,R | C,D3,H,R | C,D3,H,R | C,H,R |

[a]Key: A - Anti-ERV neutralizing antibody titer determination,

B - Brucella antigen inoculation,

C - am and pm plasma cortisol determination,

D4- dexamethasone (4 mg) administration,

D3- dexamethasone (3 mg) administration,

D2- dexamethasone (2 mg) administration,

D1- dexamethasone (1 mg) administration,

E - anti-brucella antibody determination,

H - hematology, plasma protein and fibrinogen determination,

I - in vitro lymphocyte studies,

L - lymph node biopsy collection,

R - rectal temperature and general clinical evaluation,

S - sheep red blood cell innoculation,

T - anti-sheep red blood antibody titer determination,

V - Equine Rhinopneumonitis Virus (ERV) innoculation,

X - Rhinomune$^{R}$ secondary vaccination.

Table 5 (Continued)

| Day | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|
| 8 | C,R | C,D2,R | C,D2,R | C,R |
| 9 | C,R | C,D1,R | C,D1,R | C,R |
| 10 | C,R | C,R | C,R | C,R |
| 11 | C,R | C,R | C,R | C,R |
| 12 | A,C,E,H,I,R,T | A,C,E,H,I,R,T | A,C,E,H,I,R,T | A,C,E,H,I,R,T |
| 13 | R | R | R | R |
| 14 | L,R,S | L,R,S | L,R,S | L,R,S |
| 15 | R | R | R | R |
| 16 | R | R | R | R |
| 17 | C,E,H,I,R | C,E,H,I,R | C,E,H,I,R | C,E,H,I,R |
| 18 | R | R | R | R |
| 19 | R | R | R | R |
| 20 | R | R | R | R |
| 21 | R | R | R | R |
| 22 | A,C,E,H,R,T | A,C,E,H,R,T | A,C,E,H,R,T | A,C,E,H,R,T |
| 35 | A,T | A,T | A,T | A,T |
| 40 | A,X | A,X | A,X | A,X |
| 50 | A | A | A | A |
| 57 | A | A | A | A |
| DAM | A,E | A,E | A,E | A,E |

2. Mares

Adult ponies and horses in experimental groups 5 and 6, respectively were sampled between 7:45 and 8:30 am and again between 5 and 5:30 pm for four-to-six days. Plasma samples were collected, processed and stored at -20°C as described previously, and later evaluated for cortisol levels.

## D. Serological Testing

1. Brucella

The macroscopic tube agglutination test (199) was used to titrate agglutinating antibody to Brucella abortus. Brucella antigen[1] was diluted 1:100 in 0.5 percent phenolized NSS. The serum or plasma samples were thawed in a 37°C water bath and then vortexed to ensure even mixing. Borosilicate glass tubes[2] (12x75 mm) were set up in rows of eleven tubes in test tube racks and numbered. NSS (0.6 ml) was pipetted into the first tube in each row and 0.5 ml in every other tube. Serum (0.4 ml) was added to the first tube and after thorough mixing, serial two-fold dilutions of serum were made by transferring 0.5 ml. The last tube in each row had no serum added and thus acted as a negative control. A high titer positive control bovine serum was included in each run. Serum dilutions of 1:5 through 1:2560 were achieved by the dilution process. Prepared tube antigen (0.5 ml) was added to each tube and mixed by gentle shaking, followed by overnight incubation in a 37°C water bath. A positive test was indicated by a visible agglutinate present at the bottom of the tube. The titer was expressed as the reciprocal of the highest dilution giving a positive reaction.

---

[1]Brucella Antigen (Tube), United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services Lab., Diagnostic Reagents, NADC, Ames, Iowa.

[2]diSPo$^{R}$ culture tubes, Kimble Division, American Hospital Supply Corp., McGaw Park, Illinois.

2. SRBC titers

A direct hemagglutination test employing the Microtiter technique[1] was used for titration of antibodies to sheep red blood cells (SRBC). Sheep blood was collected aseptically into an equal volume of Alsever's solution and stored at 4°C. A 3-4 ml aliquot of this suspension was washed three times in NSS, as previously described, before each assay. The washed cells were resuspended in NSS and adjusted to a final concentration of approximately .5% for use in the assay.

The sera to be tested were thawed in a 37°C water bath, followed by vortexing to ensure good mixing. Serial two-fold dilutions in a .05 ml volume of NSS were made in microtiter plates starting with a 1:2 dilution and progressing to a 1:2056 dilution. The final well in each row had no serum added and served as a negative control. One drop (0.05 ml) of the SRBC suspension was added to each well and mixing was accomplished by scratching the bottom of the plates. The plates were incubated at room temperature for one hour before being read. A positive test was indicated by a diffuse mat of cells covering the bottom of the microtiter plate well and a negative test by aggregation of the cells into a dense button at the bottom of the well. The titer was expressed as the reciprocal of the highest dilution giving a positive reaction.

## E. In Vitro Lymphocyte Studies

1. Lymphocyte separation

Lymphocyte separation was accomplished using a modification of the method of Böyum (29). Ficoll-Hypaque was prepared by mixing 80 ml of a 10% solution of Ficoll[2] in distilled water with 30 ml of 50% Hypaque[R][3]. The specific gravity of the mixture was adjusted to 1.081 with distilled water using a hydrometer, followed by sterilization of this solution by

---

[1] Microtiter[R], Cooke Engineering Co., Alexandria, Virginia.

[2] Ficoll[R], Sigma Chemical Co., St. Louis, Missouri.

[3] Hypaque[R], Winthrop Laboratories, Division of Stirling Drug Inc., New York, New York.

filtration.

Hank's balanced salt solution (HBSS) was prepared by making a 1:10 dilution of commercially available concentrated HBSS (without calcium, magnesium, and sodium bicarbonate)[1] in distilled water, followed by the addition of 0.35 g per liter of tissue culture grade sodium bicarbonate[2].

Defibrinated blood, collected as previously described, was aseptically transferred to sterile screw-capped 50 ml culture tubes which were then placed at a 45° angle in a 37°C water bath for about 20 minutes. Further settling of erythrocytes was achieved by positioning the tubes vertically for an additional 15-20 minutes. Lymphocyte-rich serum (8-10 ml) was then harvested and layered onto 3 ml of Ficoll-Hypaque in sterile screw-capped 16x125 mm polystyrene culture tubes[3] followed by centrifugation at approximately 250xg for 20 minutes. The lymphocyte band was then harvested from each tube, using a glass pipette and pro-pipette, and transferred to 50 ml tissue culture tubes to which 40 ml of warm HBSS was added. After centrifugation at 200xg for 10-15 minutes, the supernatant was decanted and the washed lymphocytes resuspended in 1 ml of warm HBSS. A 0.1 ml aliquot of this suspension was removed for counting on a hemocytometer.

2. Lymphocyte culturing

The tissue culture medium (TCM) was prepared by addition of 25 m moles of Hepes[4] and 2.2 g per liter of TC grade sodium bicarbonate to medium 199 with Earl's salts and L-Glutamine.[5] To 100 ml of this medium was added 20 ml of normal horse serum and 1 ml of an antimicrobial preparation.[6] Mitogen preparations were obtained from commercial sources

[1]Hank's balanced salt solution (10 x concentration), Grand Island Biological Co., Grand Island, New York.

[2]Sodium bicarbonate, Grand Island Biological Co., Grand Island, New York.

[3]Corning 25200 Tissue Culture Tube, Corning Glass Works, Corning, New York.

[4]Hepes, Grand Island Biological Co., Grand Island, New York.

[5]Medium 199, Grand Island Biological Co., Grand Island, New York.

[6]Antibiotic-Antimycotic Mixture, Grand Island Biological Co., Grand Island, New York.

and diluted with HBSS. Pokeweed mitogen (PWM)[1] was used in a 1:10 dilution, Concanavalin A (Con A)[2] in a 1:100 dilution (.5 mg/ml) and Phytohemagglutinin (PHA)[3] in a 1:200 dilution. PHA dilutions of 1:50, 1:100, and 1:400 were also employed in some of the initial studies, but the 1:200 dilution was used from then on since it was found to give optimal stimulation. An anti-globulin preparation, obtained from rabbits immunized with equine gamma globulin, was titrated for optimal activity and used in a 1:10 dilution. ERV antigen for use in lymphocyte cultures was prepared by high speed centrifugation of the medium from rabbit kidney cells infected with the Army 183 strain of ERV, followed by heating at 56°C for one hour to inactivate the virus. After further centrifugation, the ERV antigen was resuspended in HBSS to an optimal concentration for stimulation of equine lymphocytes and 0.1 ml was added to the cultures.

After counting, the washed lymphocytes were standardized to a concentration of $10^6$ cells/ml in TCM. This suspension (1 ml) was then transferred to 16x100 mm disposable glass culture tubes[4] with Morton closures. Volumes (0.1 ml) of the mitogen, antiglobulin or virus preparations were added to the respective experimental cultures, while the remaining tubes served as controls with nothing added. Virus tubes were run in triplicate and incubated in a 37°C incubator with a 5% $CO_2$ enriched atmosphere for four days. Mitogen and antiglobulin stimulated cultures were run in duplicate or triplicate, depending on the lymphocyte yield, and incubated as above for 48 hours. Incubation was followed by the addition of 0.1 ml (1 µc) of $^3$H-thymidine[5] to each tube and further incubation for twenty hours.

---

[1] Pokeweed mitogen, Grand Island Biological Co., Grand Island, New York.

[2] Concanavalin A, Miles-Yeda, Ltd., Miles Laboratories, Kankakee, Ill.

[3] Phytohemagglutinin$^P$, Difco Laboratories, Detroit, Michigan.

[4] B-D$^R$, RTU Techniglas, Becton Dickinson and Co., Rutherford, New Jersey.

[5] $^3$H-Thymidine, New England Nuclear, Boston, Mass.

3. Harvesting and counting

Three ml of tissue culture PBS was added to each tube followed by centrifugation at 1000xg for 10 minutes in a refrigerated centrifuge. The supernatant fluid was aspirated and the pellet frozen in a -20°C freezer. One drop of normal bovine serum was added to each tube after removal from the freezer and they were prepared for counting by two precipitations with 5% trichloroacetic acid followed by drying in a 72°C oven. The pellets were resolubilized in .5 ml Soluene$^{R}$ 350[1] and 6 ml of scintillation fluid[2] was added to each tube. The contents were transferred to labeled scintillation vials and the tubes washed with a further 6 ml of scintillation fluid. The vials were counted in a liquid scintillation counter[3] and the total counts per minute (cpm) recorded. Net counts per minute were calculated by subtraction of the cpm for unstimulated cultures (background) from the cpm of stimulated cultures. The stimulation index (SI) was derived by division of the total cpm by the background cpm.

## F. Cortisol Determination

Plasma cortisol determinations were made using a commercially available ($^{125}$I) radioimmunoassay (RIA) kit procedure[4] designed for use on human plasma or serum. A slight modification in the manufacturer's protocol was necessary since equine cortisol levels, especially in animals suppressed with DXM, were found to be lower than those reported in man (93,174,194). This kit procedure was chosen from many currently available on the basis of the following:

(i) Only a small volume (100-200 µl) of plasma was needed in the assay.

(ii) The reaction conditions (pH 5.1) caused denaturation of cortisol binding globulin and sodium salicylate contained in the reaction

---

[1]Packard Instrument Company Inc., Downers Grove, Illinois.

[2]Permaflour$^{R}$, Packard Instrument Co., Inc., Downers Grove, Illinois.

[3]Packard Tri-Carb, Liquid Scintillation Spectrometer, Packard Instrument Co. Inc., Downers Grove, Illinois.

[4]($^{125}$I) Cortisol RIA PREMIX Kit, Diagnostic Products Corporation, Los Angeles, California.

buffer blocked binding by albumin, alleviating the need for time-consuming heat denaturation, organic solvent extraction, or chromatographic isolation steps.

(iii) Use of the "Premix" method minimized the potential technical error by reducing the number of crucial pipetting steps and also cut down on the time needed for the assay.

(iv) The vendors had thoroughly validated the kit for precision, specificity, sensitivity, and effects of interfering proteins and the results were reported in the assay protocol.[1] Excellent within and between-run precision had been established over a wide range of values. There was no observable end of run effect and no effect due either to hemolysis of samples or their plasma protein content.[1]

(v) The assay was shown to be very specific for cortisol with extremely low cross-reactivity with other naturally occurring steroids or DXM.[1]

1. Preparation of reagents

All reagents were prepared at least thirty minutes before use and stored at 4°C.

(i) Citrate buffer was reconstituted with 500 ml of distilled water in a glass flask and dissolved with the aid of a magnetic stirrer. The pH of the diluted buffer was approximately 5.1.

(ii) Six vials containing Sigma grade lyophilized hydrocortisone[2] were reconstituted with 1 ml of distilled water to produce calibrators representing respectively 0, 1, 5, 10, 20, and 50 µg of cortisol/dl. An additional 0.5 µg/dl calibrator was prepared as needed by mixing equal volumes of the 0 and 1 µg/dl solutions.

(iii) A solution of polyethylene glycol (PEG) was prepared by dissolving one 15 g vial of PEG, as supplied, in 250 ml NSS followed by magnetic stirring in a glass flask.

---

[1] ($^{125}$I) Cortisol RIA PREMIX Kit, Diagnostic Products Corporation, Los Angeles, California.

[2] Sigma Chemical Company, St. Louis, Missouri.

(iv) The lyophilized ($^{125}$I) cortisol supplied had been prepared by radio-iodination at the 3-position of the steroid molecule using the chloramine-T method. This preparation was reconstituted in the vials provided by addition of 11 ml of citrate buffer followed by gentle swirling to dissolve the contents.

(v) Lyophilized anti-cortisol antiserum was reconstituted with 10 ml of Citrate buffer as above, the final solution containing 0.05% sodium azide to suppress bacterial growth. This antiserum had been raised in rabbits against cortisol conjugated with a carrier protein at the 3-position of the steroid nucleus.

(vi) Lyophilized salt-precipitated and purified goat anti-rabbit gamma globulin was dissolved in 11 ml of Citrate buffer in the vials supplied.

2. "Premix" method for preparation of the calibration curve

(i) Plasma samples were thawed in a 37°C water bath and thoroughly vortexed to dissolve any plasma clots.

(ii) 12x75 mm glass culture tubes[1] were placed in test tube racks and labeled in duplicate S1 to S6, MB (maximum binding), B (blank), and T (total counts). Additional tubes for control and unknown samples were also appropriately labeled.

(iii) The seven calibrator and control samples (apart from the pooled suppressed control) were diluted 1:31 by addition of 100 µl of sample to 3 ml of citrate buffer in 16x100 mm glass tubes. Unknown samples and the pooled suppressed equine control were diluted 2:31 (200 µl sample in 2.9 ml buffer). Pipetting was achieved using an automatic pipette[2] with a clean disposable plastic tip[3] for each sample.

---

[1] diSPo$^{R}$ Culture Tubes, Kimble Division, American Hospital Supply Corp., McGaw Park, Illinois.

[2] MLA "Selectable" Precision Pipette, Medical Laboratory Automation Inc., Mount Vernon, New York.

[3] MLA Disposable Pipette Tips, Medical Laboratory Automation Inc., Mount Vernon, New York.

(iv) 100 ml of the 0 µg/dl calibrator was pipetted into the MB and B tubes and the remaining tubes S1 to S6 were set up by adding 100 µl of the 50, 20, 10, 5, 1, and .5 µg calibrators, respectively. 100 µl of diluted control and experimental samples were similarly pipetted into their respective tubes.

(v) 100 µl of reconstituted ($^{125}$I) cortisol was added to the T- and B-tubes and 100 µl of the goat anti-rabbit gamma globulin was added to the B-tubes only.

(vi) The "Premix" was then prepared by combining equal parts of the labeled cortisol, cortisol antibody and the second antibody solutions and stored at 4°C. Before and during use, this premix was thoroughly stirred using a magnetic stirrer at room temperature since it tended to turn cloudy on storage.

(vii) An automatic pipette[1] with disposable plastic tips[2] was used to add 300 µl of the Premix to all tubes except the B- and T-tubes. After incubation in a 37°C water bath for one hour, the tubes were allowed to stand at room temperature for fifteen minutes. 2 ml of ice cold 6% PEG saline solution was then added to each tube (except T-tubes) to aid precipitation of the immune complexes, followed by centrifugation at 1,500xg for twenty minutes in a refrigerated centrifuge.[3]

(viii) The supernatant fluid was decanted and the rim of the tube carefully blotted prior to counting for two minutes in a gamma counter.[4]

(ix) Results were tabulated and duplicate determinations averaged to give average counts per minute (cpm). Subtraction of blank tube counts gave net cpm. The percentage bound radioactivity was calculated by dividing the respective sample net cpm by the net cpm of the maximum binding (MB) tubes. A standard curve was plotted using calculated

---

[1]Quickpette, Helena Laboratories, Beaumont, Texas.

[2]Cole Palmer Instrument Co., Chicago, Illinois.

[3]IEC PR-600, Damon/IEC Division, International Equip. Co., Boston, Mass.

[4]Iodine-125 Biogamma II, Beckman Instruments, Fullerton, California.

calibrator values on the logit-log paper provided; percentage bound being the ordinate and µg/dl cortisol being the abscissa. Control and sample values were then read from this curve and the results halved for those samples with a initial dilution of 2:31 rather than 1:31.

The kit was validated for equine use by comparing values for dichloromethane (DCM)-extracted and untreated equine plasma, by spiking and recovery studies, and by evaluation of parallelism between values from different dilutions of a given plasma sample. Control equine plasma samples and commercially available controls[1] were run with each assay to determine the inter-assay variability.

3. Solvent extraction procedure

(i) 200 µl of plasma was vortexed with 3 ml DCM in 16x100 mm glass tubes for one minute followed by the addition of 1 ml of distilled water and vortexing for one further minute.

(ii) Tubes were incubated at 37°C for five minutes followed by centrifugation at 1000xg for ten minutes in a refrigerated centrifuge.

(iii) The upper aqueous layer was aspirated and the solvent layer washed with 2 ml NSS, vortexed, centrifuged, and the aqueous layer aspirated as above.

(iv) The DCM solvent layer was dried in a stream of air and the solute redissolved in 3.1 ml of citrate buffer by thorough vortexing.

(v) 100 µl of this solution was then assayed along with diluted unextracted plasma and the results compared.

4. Spiking experiments

Two spiking experiments were performed to investigate the accuracy of the test procedure and its sensitivity for added cortisol (1). In the first experiment, 100 µl of the 20 µg cortisol calibrator was added to five diluted equine control plasma samples containing 100 µl of the sample in 2.9 ml of citrate buffer. Aliquots (100 µl) of these

[1] Dade® Chemistry Control, Dade Division, American Hospital Supply Corporation, Miami, Florida.

spiked samples were assayed along with unspiked controls and the percentage recovery calculated by dividing the assayed value by the expected combined value determined by summation of the assayed raw value +20 µg.

In a second experiment, equine plasma samples were spiked with a Dade III cortisol standard,[1] otherwise the procedure was as described above.

5. Parallelism experiment

The following experiment was designed to investigate the effects of equine plasma proteins on the assay. Seven equine plasma samples were assayed in three dilutions: 1:31, 1:16, and 1:11 by diluting 100, 200, and 300 µl, respectively, of plasma in 3 ml of citrate buffer during the initial dilution stage. Assayed values for the concentrated samples were divided by the value expected based on the assayed values of the unconcentrated samples and the degree of dilution, the results being expressed as percentage recovery.

## G. Lymph Node Biopsy Evaluation

Sections of formalin-fixed lymph nodes were stained with either hematoxylin-eosin or methyl green-pyronine-Y for histological examination. Attempts were made to quantitate the activity in the lymph nodes as follows:

1) Blast forms - an estimate of the number of immature lymphoid cells in the cortical and paracortical areas:

\+ = 15-20%
++ = 20-35%
+++ = 35-50%
++++ = above 50%

2) Follicle numbers - the number of primary or secondary lymphoid follicles visible in a typical cortical view as seen with a 10 x objective.

---

[1] Dade® Chemistry Control, Dade Division, American Hospital Supply Corp., Miami, Florida.

3) General - general observations of the lymph node and an estimate of mitotic figures in follicular and paracortical areas (reported as mitotic figures per high power field).

## H. Clinical Treatments and Postmortem Examinations

With the exception of foal 3077, none of the foals received any treatment during the course of the investigation, even though some became ill. Foal 3077 was given milk by stomach tube and two million units of procaine penicillin G along with 2,500 mg dihydrostreptomycin[1] by intramuscular injection the day before it died.

The three animals which died during the study (JP77, 3077, 2377) were subjected to a routine postmortem examination and the following tissues were collected for histological examination; brain, liver, kidney, adrenal, spleen, thymus, turbinate, trachea, lung, intestine, esophagus, mandibular lymph node, bronchial lymph node, mesenteric lymph node and popiteal lymph node. Affected organs were sampled for bacterial and viral isolation.

## I. Statistical Methods

The data accumulated during the course of this investigation were examined to determine trends with increasing age, and the effects of dexamethasone and equine rhinopneumonitis virus treatment on the various parameters studied. In general, for each comparison made, two of the four groups served as control animals. The values for control animals were averaged and a mean and standard error of the mean were calculated for each time period studied. Similarly, mean values and standard errors of the mean's were calculated for experimental animals. An analysis of variance procedure (196) was then performed to determine the level of significance of differences between control and experimental foals so that the influence of the above treatments on the parameters studied could be determined.

[1]Combiotic$^{R}$, Pfizer Inc., New York, New York.

The effect of age on cortisol concentrations and certain hematologic values was determined by comparing mean values for control foals of different ages using the analysis of variance procedure (196). In some cases, the values on a number of days were averaged and the mean compared to the mean pooled value for another age group.

# IV. RESULTS

## A. Validation of the Cortisol Assay Procedure

The kit procedure used for cortisol determinations had previously been validated by the vendor, on the basis of sensitivity, specificity, accuracy and precision, for use on human plasma. It had also been shown that plasma proteins did not significantly interfere with the assay, eliminating the need for extraction of the cortisol from the sample prior to assay.[1] To further evaluate the suitability of the kit for use on equine plasma, four validation experiments were performed.

### 1. Extraction experiment

One human and four equine control plasma samples were extracted with dichloromethane and assayed in the same run as the corresponding nonextracted samples. The recovery percentages were calculated by dividing the values for assayed concentrations of extracted samples by those of the respective nonextracted samples (Table 6). The mean recovery rate for equine samples was 95%, indicating that this kit procedure is well-suited for use on equine plasma samples without the need for prior solvent extraction.

Table 6. Effect of solvent extraction on plasma cortisol concentrations

| Sample source | Sample # | Cortisol concentration (µg/dl) Nonextracted | Extracted | Recovery (%) |
|---|---|---|---|---|
| Human | 111S | 4.8 | 5.5 | 114.6 |
| Equine | OB 16 | 3.8 | 3.6 | 94.7 |
| Equine | PS | 0.8 | 0.65 | 81.3 |
| Equine | OB 14 | 4.9 | 5.8 | 118.4 |
| Equine | OB 17 | 6.8 | 5.8 | 85.3 |
| | | | MEAN (Equine samples) | 94.93 |
| | | | MEAN (All samples) | 98.86 |

[1] Assay Protocol, [$^{125}$I] Cortisol RIA "Premix" Kit, Diagnostic Products Corp., Los Angeles, CA.

2. Spiking and recovery experiments

Two experiments were conducted to assess the accuracy of the assay procedure, over a range of cortisol concentrations, by determining the recovery of added cortisol. In the first experiment (A), three equine plasma samples were "spiked" with an equal quantity of the 111S human control and the reaction volumes adjusted accordingly. "Spiked" and corresponding "nonspiked" samples were quantitated during a single assay run and the percentage recovery of added cortisol was calculated as shown in Table 7.

Table 7. Evaluation of assay accuracy - experiment A

| Sample | Cortisol concentration (µg/dl) | | | Recovery (%)[a] |
|---|---|---|---|---|
| | Assayed nonspiked | Assayed spiked | Expected spiked | |
| OB 16 | 3.8 | 7.6 | 8.6 | 88.4 |
| OB 14 | 4.9 | 12.0 | 9.7 | 123.7 |
| OB 17 | 6.8 | 12.8 | 11.6 | 110.4 |
| 111S | 4.8 | | | |
| | | | MEAN | 107.5 |

[a] $\frac{\text{Assayed concentration}}{\text{Expected concentration}} \times 100\%$.

In the second experiment (B), five equine samples were "spiked" with a 20 µg/dl cortisol standard; otherwise the procedure was as described in experiment A. Results are shown in Table 8.

When the recovery percentages for the individual samples quantitated in experiments A and B were combined, the mean was 99.3%. These results indicated that this assay procedure possessed a high degree of accuracy over the wide range of cortisol concentrations examined. There was, however, a slight tendency to underestimate values at the high end of the calibration curve and to overestimate values at the low end of the curve.

Table 8. Evaluation of assay accuracy - experiment B

| Sample | Cortisol concentration (µg/dl) | | | Recovery (%)[a] |
|---|---|---|---|---|
| | Assayed nonspiked | Assayed spiked | Expected spiked | |
| Pooled Eq. | 6.0 | 25.0 | 26.0 | 96.2 |
| Billy | 4.7 | 24.0 | 24.7 | 97.2 |
| OB 13 | 5.2 | 25.0 | 25.2 | 99.2 |
| OB 16 | 5.0 | 21.0 | 25.0 | 84.0 |
| OB 257 | 7.2 | 26.0 | 27.2 | 95.6 |
| | | | MEAN | 94.4 |

[a] $\frac{\text{Assayed concentration}}{\text{Extracted concentration}} \times 100\%$.

3. Parallelism experiment

This experiment was carried out to determine the ability of the assay procedure to measure the cortisol concentrations of unknown samples from different parts of the calibration curve. In addition, the effect of increasing plasma protein concentrations on assay performance could be assessed. Five samples were assayed in dilutions of 1:31 (the recommended dilution), 2:32, and 3:33 and these latter values were compared with those expected by extrapolation from the concentration found in the 1:31 dilution. These results are shown in Table 9 and demonstrate a slight tendency toward increased sensitivity in samples assayed at half the recommended plasma dilution (2:32). There was no apparent interference due to plasma proteins at the 2:32 dilution but such an effect may have occurred at the 3:33 dilution since cortisol concentrations were lower than expected in all samples assayed at this dilution.

Based on these results and the fact that other workers have reported that equine plasma cortisol concentrations are generally less than 6 µg/dl (28,97,119,124), it was decided to assay all samples at the 2:32 dilution since this appeared to increase accuracy and sensitivity without apparent interference from plasma proteins.

Table 9. Effect of sample dilution on assayed cortisol concentrations

| Sample | Sample dilution | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1:31 | 2:32 | | | 3:33 | | |
| | A[a] | A[a] | E[b] | A/E% | A[a] | E[b] | A/E% |
| 111S | 4.8 | 11.0 | 9.9 | 111.1 | 15.0 | 15.3 | 98.0 |
| OB 16 | 3.8 | 8.0 | 7.9 | 101.9 | 9.4 | 12.1 | 77.7 |
| PS | 0.8 | 1.6 | 1.64 | 97.6 | 2.5 | 2.55 | 98.0 |
| OB 14 | 4.9 | 11.0 | 10.1 | 108.9 | 14.8 | 15.6 | 94.9 |
| OB 17 | 6.8 | 14.4 | 14.0 | 102.9 | 17.0 | 21.7 | 78.3 |
| MEAN | | | | 104.5 | | | 89.4 |

[a]Assayed cortisol concentration (µg/dl).

[b]Expected cortisol concentration (µg/dl).

4. Evaluation of assay precision

Interassay precision was determined by the inclusion of control equine and human samples in each assay run, followed by calculation of the coefficients of variability for each sample between different runs (Table 10).

The mean coefficient of variability for all samples was 17.42%. This value, while still very acceptable, is slightly higher than the ideal and may reflect the fact that most of the control samples used had relatively low plasma cortisol concentrations and were thus read from near the bottom of the calibration curve. It has already been noted in the parallelism experiment that the accuracy of the assay is slightly reduced at low plasma cortisol levels. In addition, the pipetting and dilution stages of the assay procedure are of crucial importance in achieving acceptable interassay precision and assume even greater significance when samples have low cortisol concentrations.

Table 10. Interassay variability in plasma cortisol determinations

| Sample | Mean[a] | S.E.M.[b] | C.V.[c] | n[d] |
|---|---|---|---|---|
| Billy | 4.64 | .32 | 24.03 | 12 |
| OB 13 | 4.20 | .24 | 16.96 | 9 |
| OB 16 | 3.96 | .32 | 24.45 | 9 |
| OB 257 | 7.23 | .20 | 4.86 | 3 |
| Pooled | 5.91 | .24 | 15.20 | 14 |
| 111S | 4.20 | .60 | 20.20 | 2 |
| 211S | 13.95 | .88 | 22.80 | 13 |
| 311S | 32.50 | 2.50 | 10.88 | 2 |
| MEAN | | | 17.42 | |

[a]Cortisol concentration (µg/dl).

[b]Standard error of the mean.

[c]Coefficient of variability.

[d]Number of samples assayed.

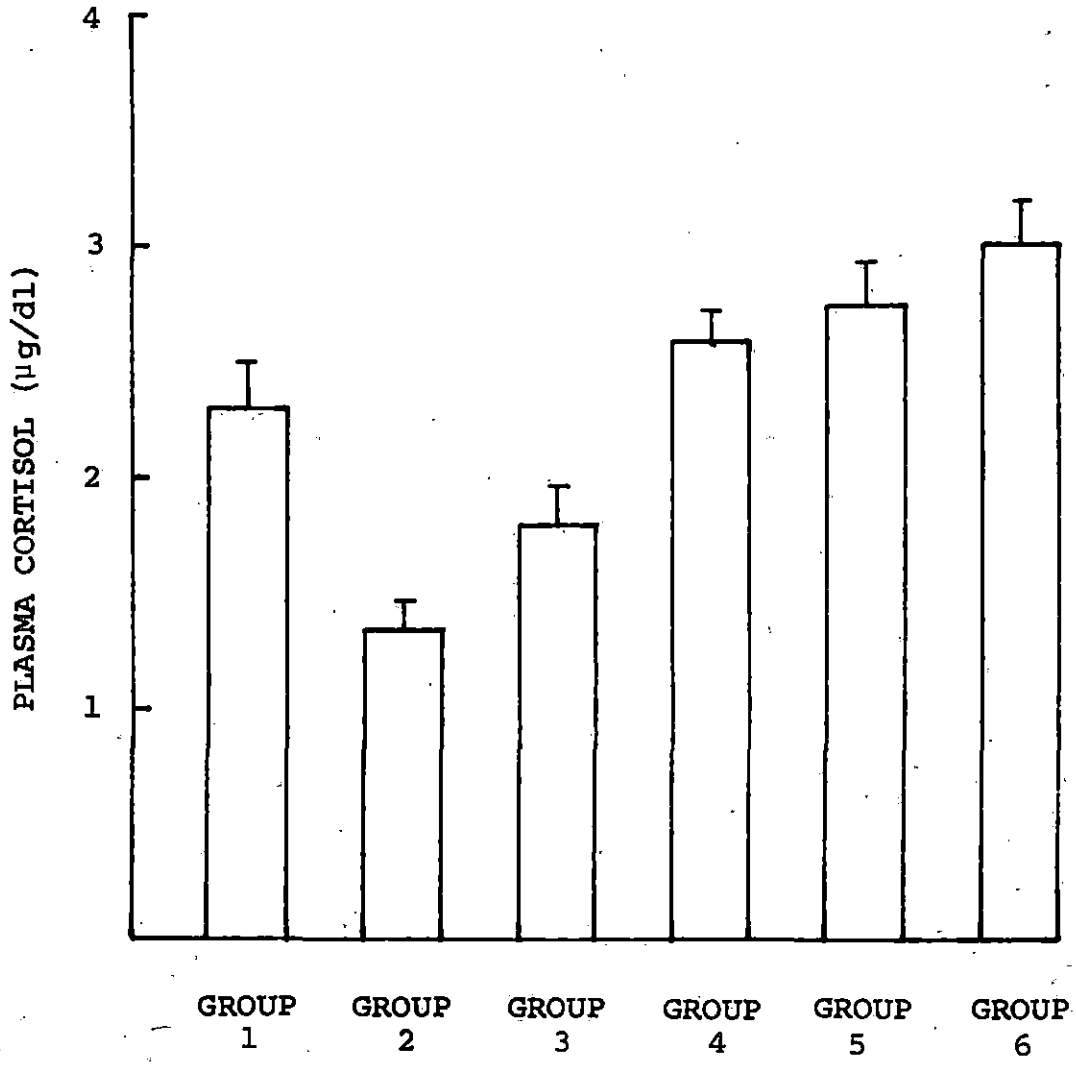
## B. Plasma Cortisol Concentrations in Foals, Adult Ponies and Horses

Plasma cortisol concentrations were measured in samples drawn twice daily at approximately 8 am and 5 pm, from foals (groups 1, 2, 3, and 4), adult ponies (group 5), and adult horses (group 6). Assayed values for adult ponies and horses served as a baseline for evaluation of age-related changes in the plasma cortisol concentrations of the young pony foals.

### 1. Adult ponies and horses

The overall mean daily plasma cortisol concentrations were calculated by averaging morning and afternoon values and it was found that levels in adult ponies (2.74 µg/dl) and adult horses (3.0 µg/dl) were not significantly different (Figure 1). There was, however, considerable

Figure 1. A histogram comparing the mean combined daily plasma cortisol concentrations (± S.E.M.) of foals over the first twenty-two days of life (groups 1, 2, 3, and 4), adult ponies (group 5) and adult horses (group 6). Animals in groups 2 and 3 received daily intramuscular injections of dexamethasone (DXM) between one and nine days of age and those in groups 3 and 4 were inoculated intravenously with equine rhinopneumonitis virus (ERV) at four days of age


PLASMA CORTISOL (µg/dl)
4
3
2
1
GROUP 1
GROUP 2
GROUP 3
GROUP 4
GROUP 5
GROUP 6

day-to-day and interindividual variation in both groups; pony values ranged from 0.9 to 7.0 µg/dl and horse values from 0.5 to 8.4 µg/dl. In addition, both groups displayed significantly higher mean cortisol concentrations in morning plasma samples than in afternoon samples ($p<0.005$). Afternoon values averaged 63.3% of morning values in ponies and 49.7% of morning values in horses. This striking diurnal variation is illustrated in Figure 2.

2. Foals from one to twenty-two days of age

Three foals (JP77, 2377, and 3077) died as a result of infectious disease during the investigation and thus their cortisol levels were not included in the results that follow.

The mean daily plasma cortisol concentrations in nondexamethasone-treated control foals are illustrated graphically in Figures 3 and 4. Levels varied considerably from day-to-day and between individuals, but there was an overall trend of decline during the first two or three days of life followed by a slow irregular rise over the next six or seven days, after which time concentrations tended to remain relatively stable. Mean daily plasma cortisol levels for various time periods during the first twenty-two days of life were compared with those of adult ponies to further demonstrate these effects, as shown in Figure 5.

Mean levels at one day of age (2.49 µg/dl) were similar to those found in adults (2.74 µg/dl) but these declined over the next three or four days so that combined concentrations in two-to-four-day-old foals (1.89 µg/dl) were significantly lower than adult concentrations ($p<0.005$). Concentrations during the five-to-nine-day-old period (2.56 µg/dl) and the ten-to-eleven-day-old period (2.27 µg/dl) were lower, and levels in twelve-to-twenty-two-day-old foals (2.98 µg/dl) were higher, than adult levels, but none of these differences were statistically significant.

When morning and afternoon plasma cortisol concentrations were plotted separately on each day (Figures 6 and 7), a distinct diurnal

Figure 2. A histogram illustrating the diurnal variation in plasma cortisol concentrations of foals (groups 1, 2, 3, and 4), adult ponies (group 5) and adult horses (group 6). Foal values are combined means for days 1 to 22. Morning (a) and afternoon (p) samples were drawn at approximately 8 am and 5 pm, respectively. Foals in groups 2 and 3 were given daily intramuscular injections of dexamethasone during the first nine days of life and those in groups 3 and 4 were inoculated intravenously with equine rhinopneumonitis virus at four days of age

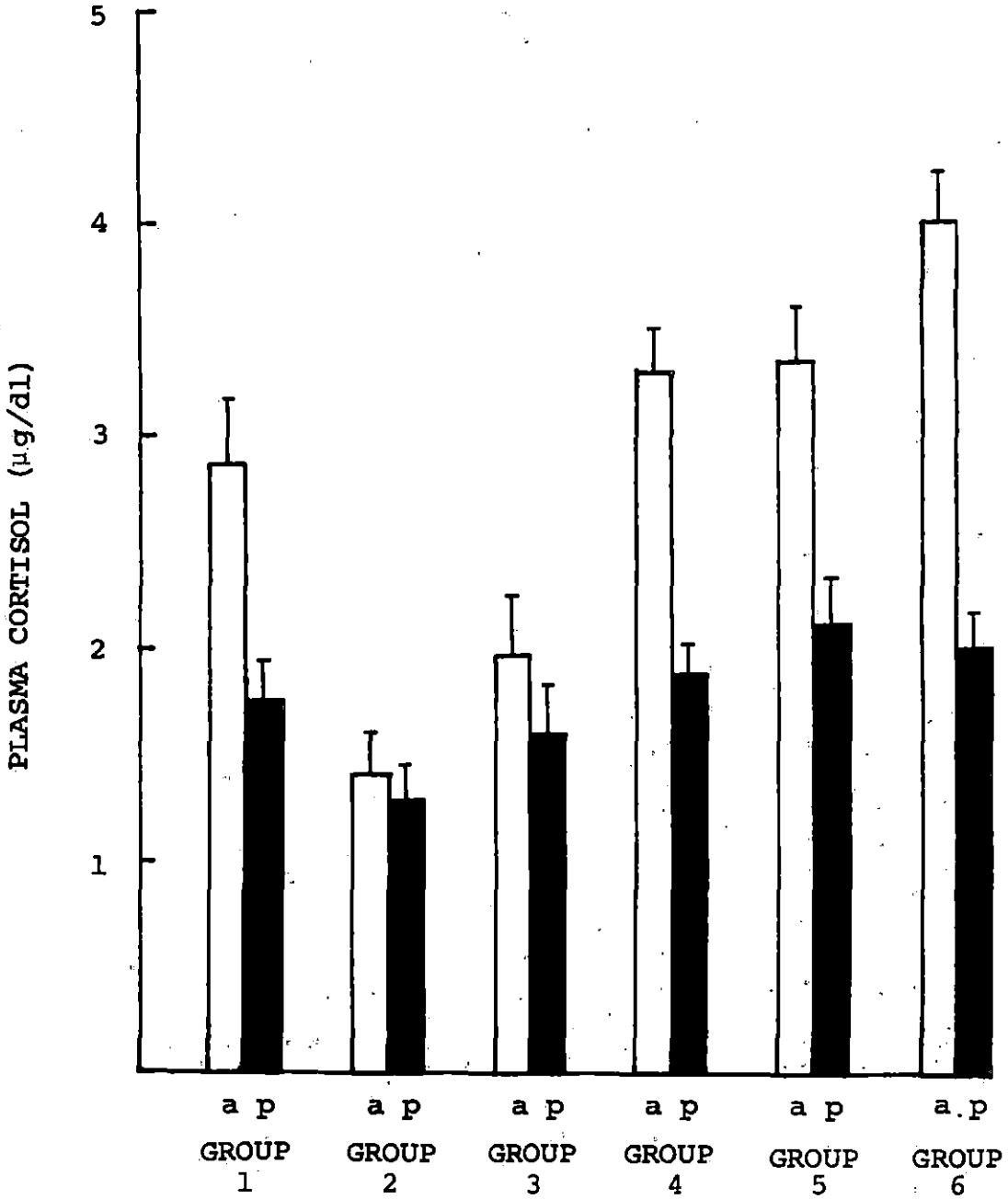

PLASMA CORTISOL (µg/dl)
5
4
3
2
1
a p
GROUP 1
a p
GROUP 2
a p
GROUP 3
a p
GROUP 4
a p
GROUP 5
a.p
GROUP 6

Figure 3. A graph illustrating the effect of age, dexamethasone (DXM), and equine rhinopneumonitis virus (ERV) on the mean daily plasma cortisol concentrations of foals. Animals in groups 2 and 3 received daily intramuscular injections of DXM during the first nine days of life and those in groups 3 and 4 were given ERV intravenously at four days of age

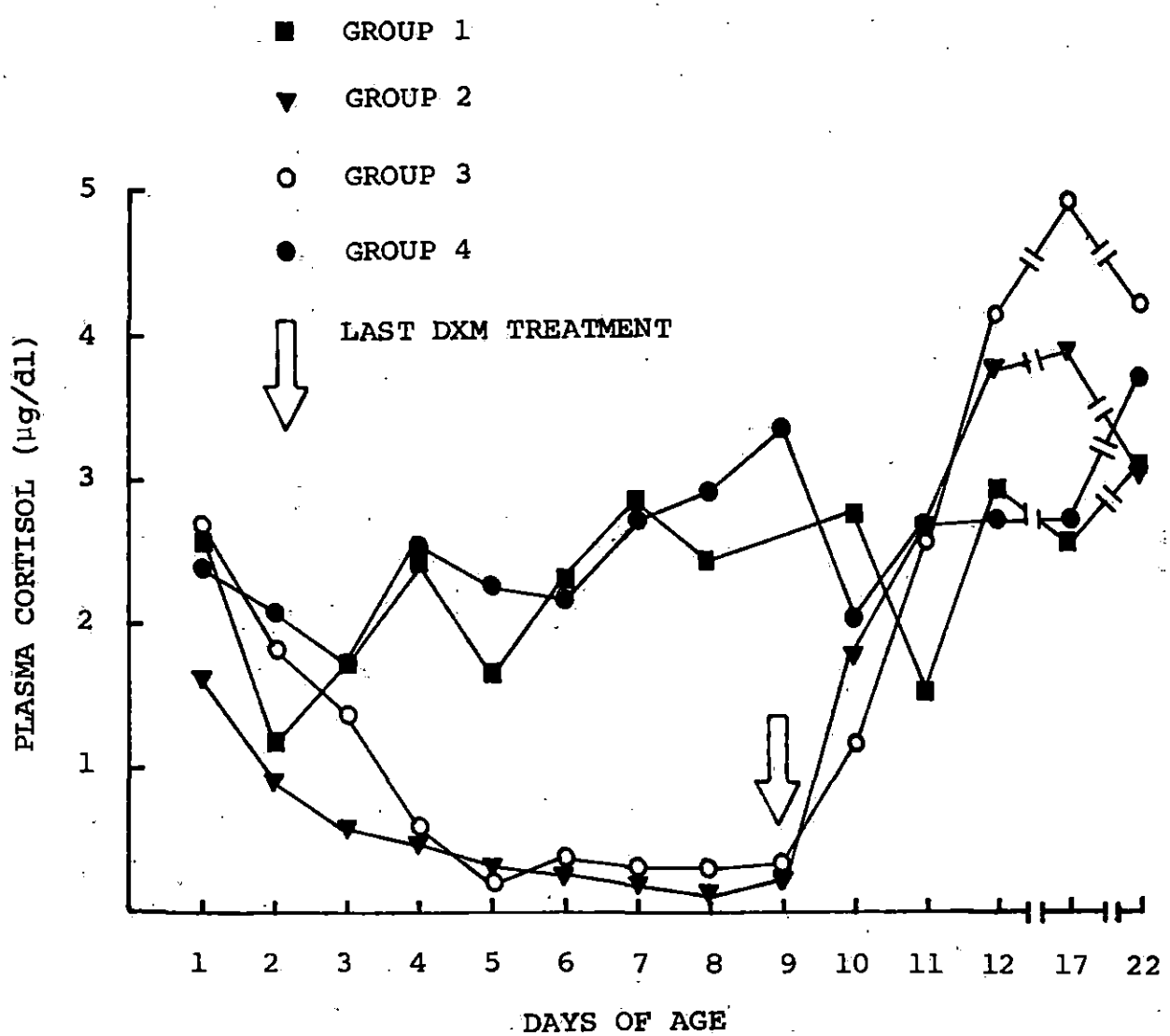

GROUP 1
GROUP 2
GROUP 3
GROUP 4
LAST DXM TREATMENT
PLASMA CORTISOL (µg/dl)
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12 17 22
DAYS OF AGE

Figure 4. A graph illustrating the effect of age and dexamethasone (DXM) therapy on the mean daily plasma cortisol concentrations of foals. Treated animals were given daily intramuscular injections of DXM during the first nine days of life

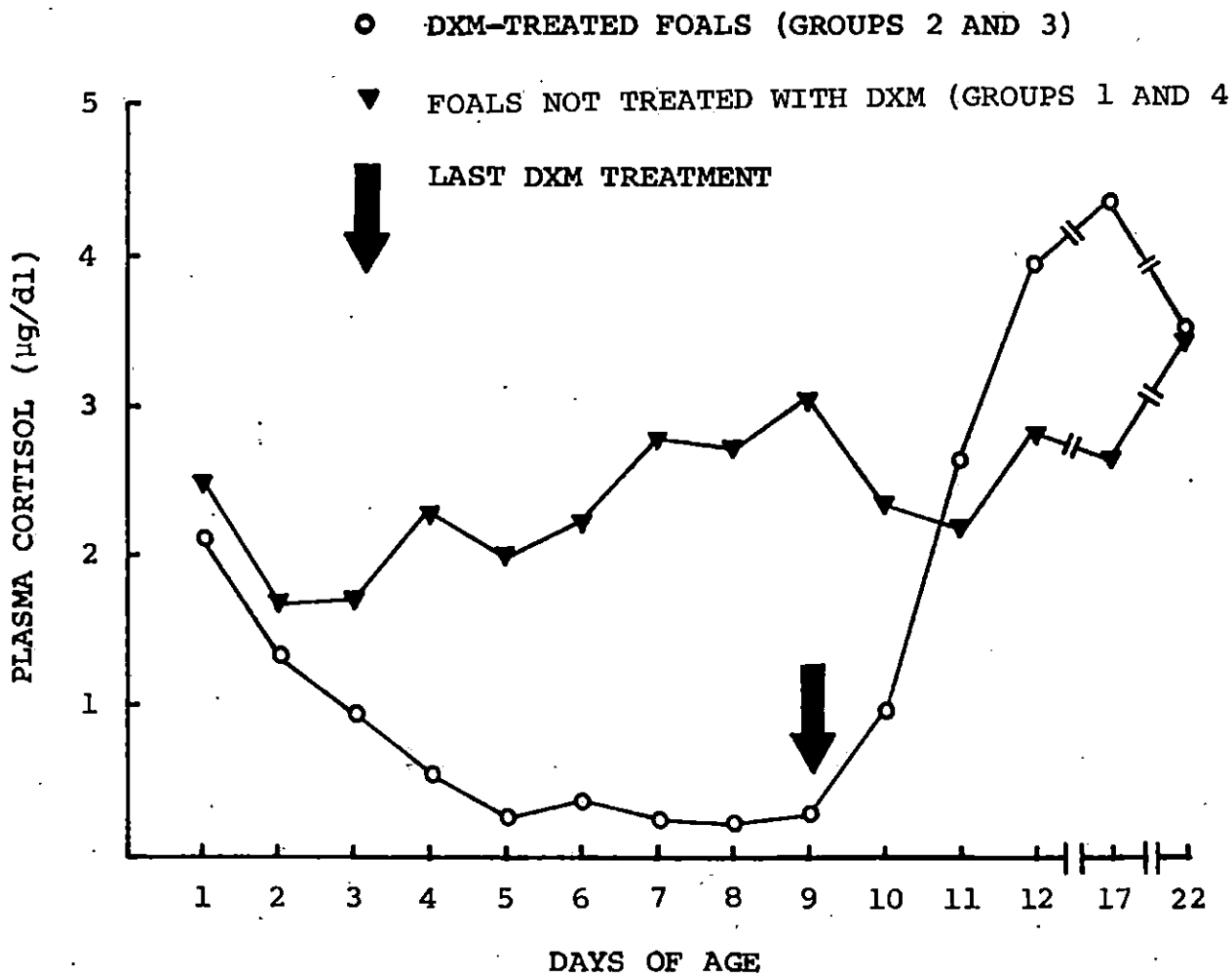

DXM-TREATED FOALS (GROUPS 2 AND 3)
FOALS NOT TREATED WITH DXM (GROUPS 1 AND 4)
LAST DXM TREATMENT
PLASMA CORTISOL (µg/dl)
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12 17 22
DAYS OF AGE

Figure 5. A histogram illustrating the age-related alterations in the plasma cortisol concentrations of foals not given dexamethasone (DXM) treatment (groups 1 and 4). Also shown is the depression in levels induced by DXM-treatment of foals in groups 2 and 3 during the first nine days of life, and the pattern of recovery following withdrawal. Cortisol concentrations in adult ponies (group 5) and adult horses (group 6) are shown for comparison

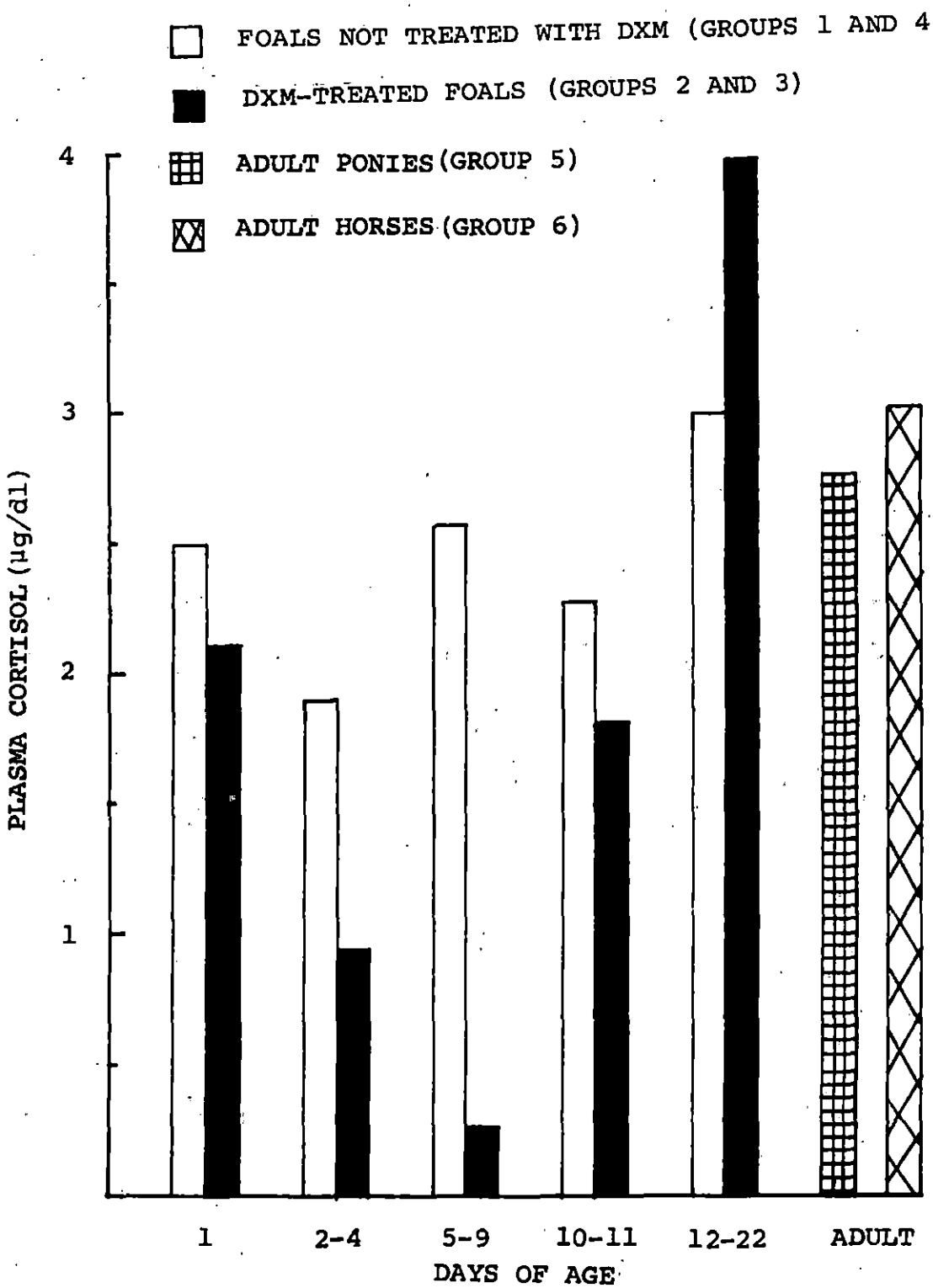

FOALS NOT TREATED WITH DXM (GROUPS 1 AND 4)
DXM-TREATED FOALS (GROUPS 2 AND 3)
ADULT PONIES (GROUP 5)
ADULT HORSES (GROUP 6)
PLASMA CORTISOL (µg/dl)
4
3
2
1
1
2-4
5-9
10-11
12-22
ADULT
DAYS OF AGE

Figure 6. A graph illustrating the effect of age, dexamethasone (DXM), and equine rhinopneumonitis virus (ERV) on the diurnal variation in plasma cortisol concentrations of foals. Animals in groups 2 and 3 received daily intramuscular injections of DXM between one and nine days of age and those in groups 1 and 4 were given ERV intravenously at four days old. Morning (a) and afternoon (p) samples were drawn at approximately 8 am and 5 pm, respectively

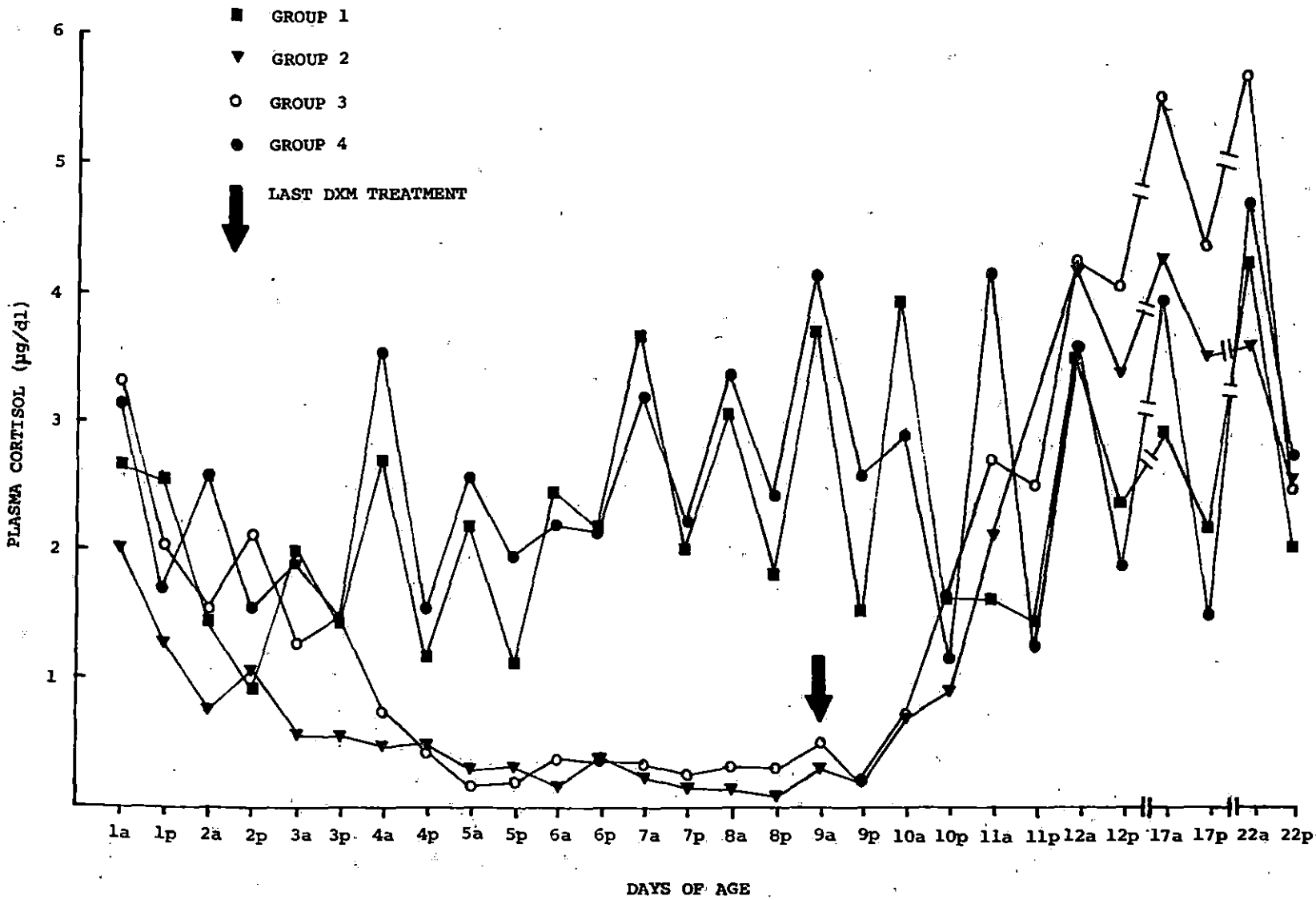
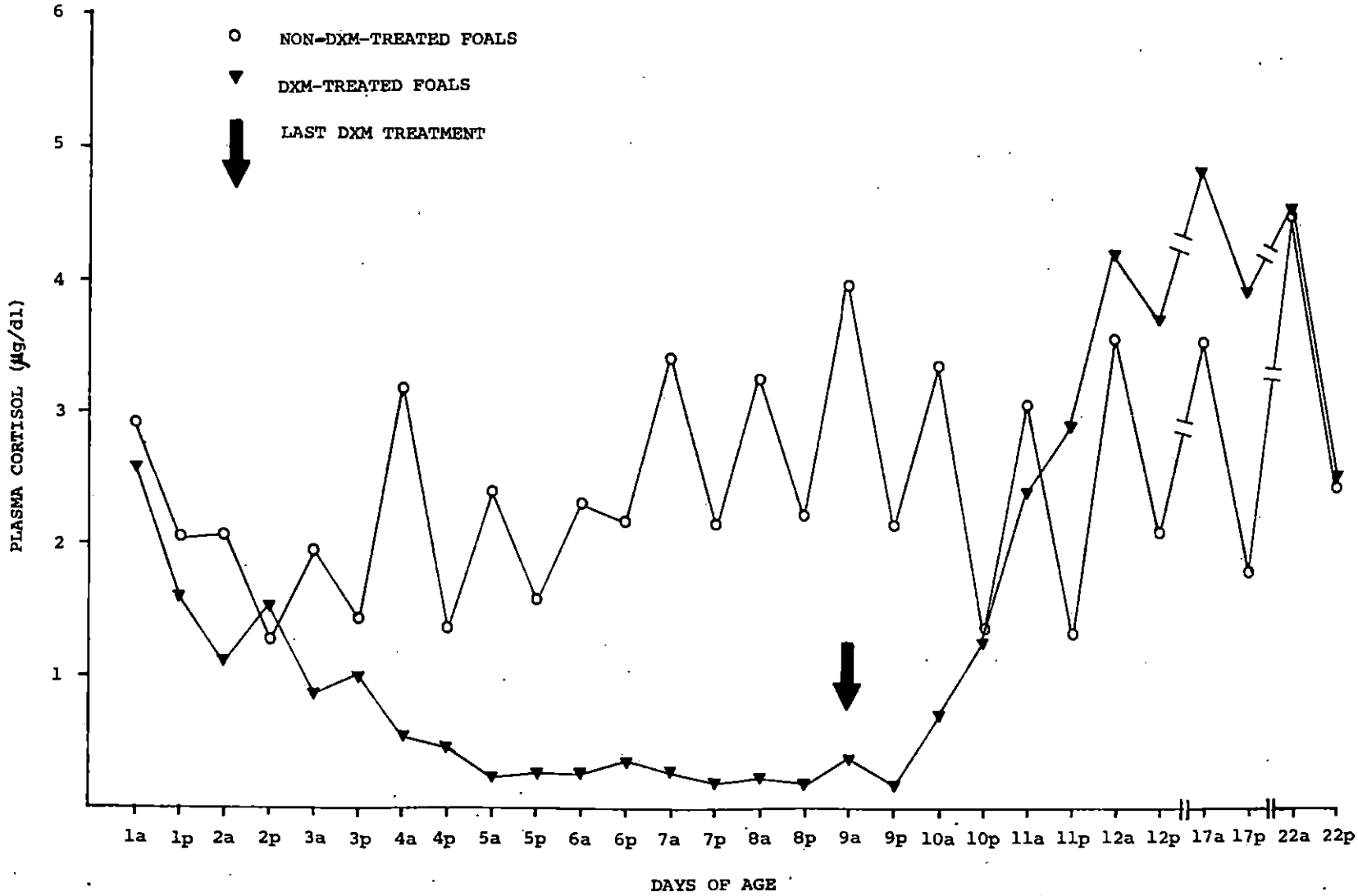

GROUP 1
GROUP 2
GROUP 3
GROUP 4
LAST DXM TREATMENT
PLASMA CORTISOL (µg/dl)
6
5
4
3
2
1
1a 1p 2a 2p 3a 3p 4a 4p 5a 5p 6a 6p 7a 7p 8a 8p 9a 9p 10a 10p 11a 11p 12a 12p 17a 17p 22a 22p
DAYS OF AGE

Figure 7. A graph illustrating the effect of age and dexamethasone (DXM) on the diurnal variation of plasma cortisol concentrations in foals. Treated animals were given daily intramuscular injections of DXM from one to nine days of age. Morning (a) and afternoon (p) samples were drawn at about 8 am and 5 pm, respectively


NON-DXM-TREATED FOALS
DXM-TREATED FOALS
LAST DXM TREATMENT
PLASMA CORTISOL (µg/dl)
6
5
4
3
2
1
1a 1p 2a 2p 3a 3p 4a 4p 5a 5p 6a 6p 7a 7p 8a 8p 9a 9p 10a 10p 11a 11p 12a 12p 17a 17p 22a 22p
DAYS OF AGE

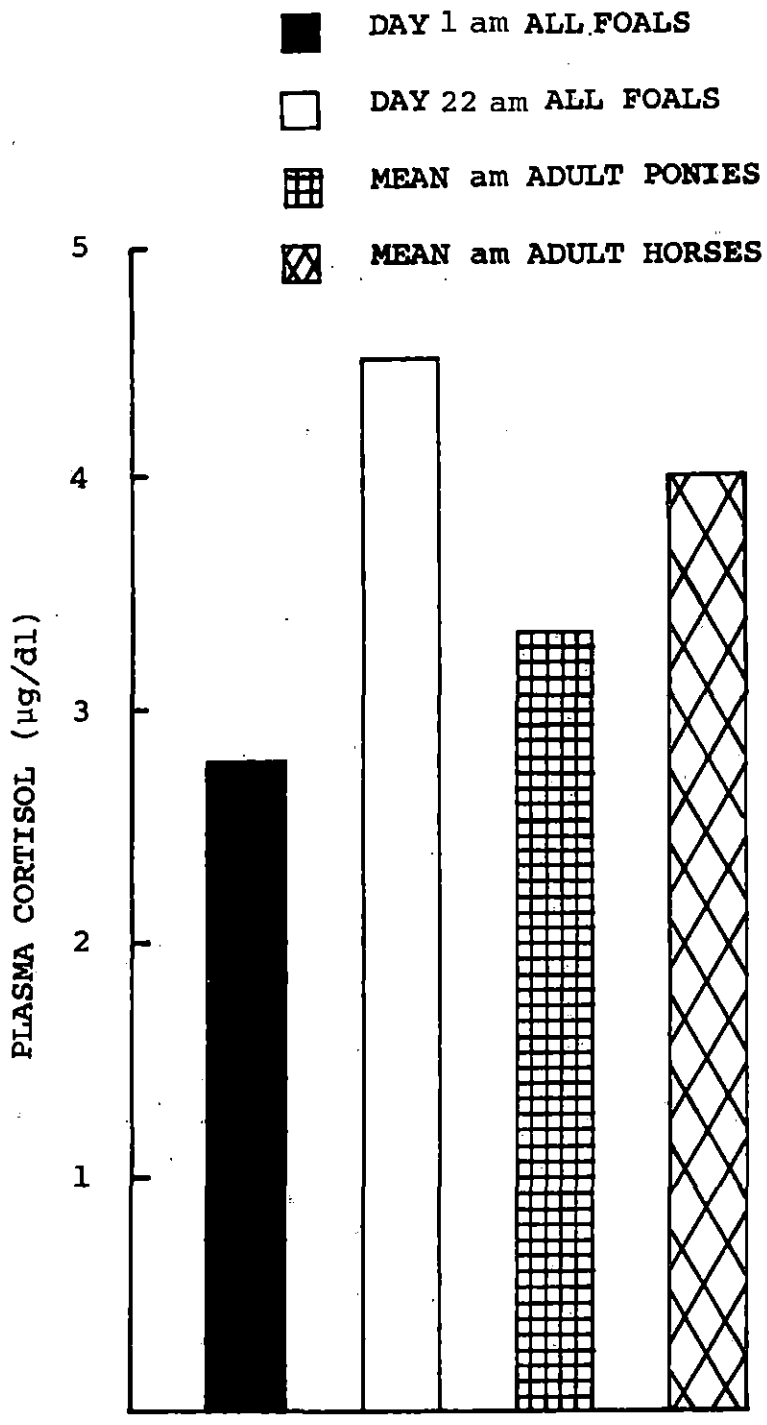
variation was observed with morning values higher than afternoon values. This pattern was already established at one day of age and remained relatively consistent throughout the investigation period, although the difference between morning and afternoon concentrations tended to become slightly more marked with increasing age. When values for all days during the first twenty-two days of life were pooled and considered together (Figure 2), it was found that control foals not treated with DXM had afternoon values which were 60.6% of those found in morning samples. This difference was highly significant ($p<0.005$) and of a similar magnitude to that observed in adult ponies.

Afternoon plasma cortisol concentrations tended to be dependent on morning levels in a rather consistent manner and thus the overall trends of fluctuation in morning levels with increasing age were very similar to those already described for mean daily concentrations. A comparison was made between the mean morning cortisol levels in one-day-old foals, twenty-two-day-old foals, and adult ponies (Figure 8). It was found that the morning levels at twenty-two days of age were significantly higher than those found in adult ponies and in one-day-old foals ($p<0.05$), while the values for the latter two groups were not significantly different from each other. These results further emphasize the trends of gradual increase in plasma cortisol levels over the first twenty-two days of life, followed sometime later by a decline to adult levels. Experimentation utilizing older foals would be necessary to further elucidate these trends.

3. Effect of dexamethasone on plasma cortisol concentrations

Foals in groups 2 and 3 received daily intramuscular injections of dexamethasone (DXM) from one-to-nine days of age. The mean daily plasma cortisol concentrations (Figures 3 and 4) were greatly reduced by DXM administration, but the maximal effect was not seen until the fifth day of this regimen. Mean daily cortisol levels remained below .30 µg/dl from the fifth to the ninth day when the last DXM treatment was given. The overall mean daily plasma cortisol concentration from the

Figure 8. A histogram illustrating the effect of age on mean morning plasma cortisol concentrations. Foal values represent the means of groups 1, 2, 3, and 4 combined


DAY 1 am ALL FOALS
DAY 22 am ALL FOALS
MEAN am ADULT PONIES
MEAN am ADULT HORSES
PLASMA CORTISOL (µg/dl)
5
4
3
2
1

second to ninth day of therapy in the DXM-treated group (.52 µg/dl) was significantly lower ($p<0.0001$) than that of nontreated foals (2.31 µg/dl) during the same period, indicating that DXM profoundly inhibits endogenous adrenocortical function.

The pattern of development of adrenal suppression was evaluated by comparing treated foals with control animals over various time periods (Figure 5). While mean daily cortisol concentrations were not appreciably reduced on the first day of therapy, they were significantly lower than those of control foals during both the second-to-fourth day and the fifth-to-ninth day periods of the DXM treatment regimen ($p<0.0001$). Levels were still depressed when the tenth and eleventh days were considered together, but "rebounded" to significantly higher concentrations than found in control animals ($p<0.05$) during the period twelve-to-twenty-two days after the first dexamethasone injection was given, indicating a rapid recovery from the suppressive effects of DXM.

The diurnal pattern of variation in plasma cortisol concentrations was eliminated by DXM treatment (Figures 6 and 7) and both morning and afternoon values were found to be significantly lower in treated foals than in untreated controls ($p<0.0001$). While control foals showed significantly lower cortisol concentrations in afternoon samples ($p<0.005$), DXM-treated animals had morning and afternoon levels which were almost identical (Table 11).

The dose of DXM administered to foals was gradually reduced over the seventh, eighth, and ninth days of treatment, but the plasma cortisol concentrations remained maximally suppressed during this time. However, morning levels had risen by the day after cessation of therapy and continued to rise through each successive sampling period for two more days, at which time the diurnal rhythm was re-established (day 12), as illustrated in Figures 6 and 7. At twelve and seventeen days of age, when mean daily plasma cortisol concentrations showed a "rebound" to supranormal levels in DXM-treated foals (Figure 4), a diurnal rhythm was present but of altered character. Cortisol levels in afternoon samples were 84% of the morning levels in treated animals as compared to 55% of morning levels in controls. This finding is further emphasized

Table 11. Effect of dexamethasone on the diurnal variation in plasma cortisol concentrations of foals from two to nine days of age

| Group | Mean plasma cortisol concentration (µg/dl) | |
|---|---|---|
| | Morning | Afternoon |
| One[a] | 2.65** | 1.52** |
| Two[b] | .37 | .41 |
| Three[b,c] | .67 | .68 |
| Four[c] | 2.94** | 2.00** |

[a]Nontreated controls.

[b]Foals given daily intramuscular injections of DXM from one to nine days of age.

[c]Foals given equine rhinopneumonitis virus intravenously at four days of age.

**Morning vs. afternoon values $p<0.01$.

by the fact that, while both morning and afternoon values were higher in treated foals at this time, the difference was only significant ($p<0.01$) for afternoon values (Table 12). This may indicate that, following the morning "cortisol surge", control foals secreted relatively small amounts of cortisol during the remainder of the day, whereas foals previously treated with DXM maintained a higher level of cortisol secretion throughout the day.

Table 12. Diurnal variation in plasma cortisol concentrations of foals during recovery from dexamethasone (DXM) suppression

| | Plasma cortisol concentration (µg/dl) - days 12 and 17 | | |
|---|---|---|---|
| | Mean morning | Mean afternoon | Mean daily |
| Treated foals | 4.51 | 3.79** | 4.15* |
| Control foals | 3.54 | 1.94** | 2.74* |

*Treated vs. control foal values $p<0.05$.

**Treated vs. control foal values $p<0.01$.

Thirteen days after the last DXM injection, i.e., day 22, both the morning and the afternoon plasma cortisol concentrations in treated foals were almost identical to those of control animals indicating full recovery from the suppressive effects of DXM (Figure 7).

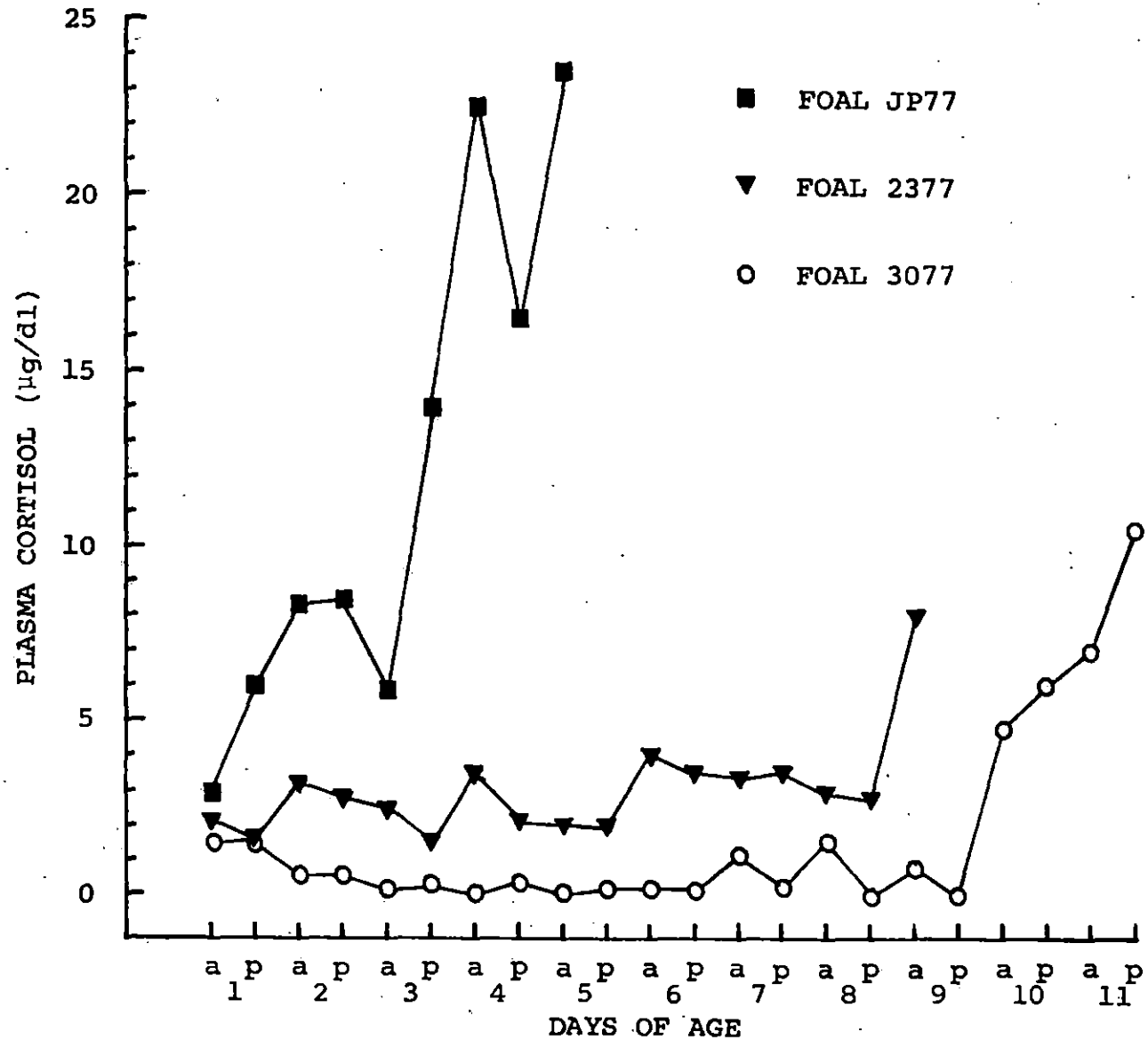
4. Effect of equine rhinopneumonitis virus on plasma cortisol concentrations

A comparison of the mean daily plasma cortisol concentrations of virus-treated and nontreated foals is shown in Figure 3. When values from one-to-twenty-two days of age were combined, it was found that the mean daily levels in virus-treated foals (2.14 µg/dl) were significantly higher ($p<0.05$) than those not given ERV (1.67 µg/dl). This statistical comparison was probably invalidated, however, by the fact that the control group contained proportionately more DXM-treated foals than the virus-treated group and it has already been shown that DXM profoundly reduces plasma cortisol concentrations. When equivalent DXM treatment groups were compared, however, higher cortisol levels were still found in the virus-treated foals (Figure 1). A similar effect was apparent when morning and afternoon values were considered separately (Figures 2 and 6), although none of the differences between virus-treated and nontreated foals reached a significant level ($p>0.05$).

5. Effect of terminal disease states on plasma cortisol concentrations

The plasma cortisol concentrations of the three foals that died during the investigation are shown in Figure 9. Foal 3077 received both DXM and ERV (group 3), while foal 2377 was a member of the virus treatment group (group 4). Both were given virulent ERV intravenously at four days of age and died acutely after showing signs of severe respiratory distress for one to two days. Foal JP77 was a control animal which became depressed at three days of age and died from an *E. coli* septicemia forty-eight hours later. A very marked terminal rise in plasma cortisol concentrations, with disruption of the diurnal rhythm, was evident in all three foals; levels approximately eight

Figure 9. A graph illustrating the terminal rise in the plasma cortisol concentrations of three foals prior to death. Foal 3077 received daily intramuscular injections of dexamethasone (DXM) between one and nine days of age and was given equine rhinopneumonitis virus (ERV) intravenously on day four. Foal 2377 was given ERV intravenously on day four, while foal JP77 was a control animal receiving no treatment


25
20
15
10
5
0
PLASMA CORTISOL (µg/dl)
FOAL JP77
FOAL 2377
FOAL 3077
a p a p a p a p a p a p a p a p a p a p a p
1 2 3 4 5 6 7 8 9 10 11
DAYS OF AGE

times higher than those of healthy animals being found in foal JP77 on the morning prior to death.

C. Clinical and Pathologic Findings

All foals were examined daily for clinical evidence of disease and rectal temperatures were recorded between 7:30 and 8 am each morning during or after sample collection.

Of the four foals in the control group (no DXM, no ERV), two showed no evidence of clinical disease and had normal rectal temperatures (less than 102°F) throughout the course of the investigation. A third foal developed a mild cough and a slightly purulent nasal discharge between fifteen and seventeen days of age and a transient diarrhea at twenty-two days of age but had a normal rectal temperature throughout the entire study. The fourth control foal (JP77) became slightly depressed at three days of age and died acutely forty-eight hours later after a rapidly progressive period of debilitation and failure to nurse. However, pyrexia was not noted during this time.

No gross lesions were observed on postmortem examination of this animal (JP77) and the only histological lesions consisted of necrosis of the submucosal lymphoid tissues of the small intestine and increased numbers of neutrophils in the small arterioles and capillaries of the lung. A diagnosis of E. coli septicemia was made on the basis of isolation of this organism in pure culture from the liver and lung. This foal had a plasma protein concentration of 5.1 g percent at one day of age as compared to a mean value of 6.55 g percent for all the other foals. Furthermore, it was the only animal lacking detectable antibodies to ERV or Brucella abortus at this time. It thus seems likely that failure of colostral antibody absorption predisposed this foal to a fatal E. coli infection.

Three foals in the ERV treatment group (group 4) were inoculated with three-to-five times the recommended dose of an attenuated live ERV vaccine[1] at four days of age. None of these animals developed any

[1] Rhinomune®, Norden Laboratories Inc., Lincoln, Nebraska.

temperature elevation or clinical evidence of disease, other than a transient diarrhea in one foal related to estrus in the dam.

The remaining two foals in this group were intravenously inoculated with virulent ERV at four days of age. Both became pyretic (rectal temperatures 103°F) forty-eight hours after viral inoculation. Two days later, one of these foals (377) became tachypneic, dyspneic, and depressed and had harsh vesicular lung sounds along with a rectal temperature of 103.8°F. These signs persisted for forty-eight hours before subsiding and by the eighth postinoculation day the rectal temperature had returned to normal and the animal again appeared clinically healthy. At seventeen days of age (thirteen days post-inoculation) a neurological disturbance became apparent and included posterior incoordination with hypermetria, dysmetria and generalized tremors. Excitement would sometimes precipitate a clonic convulsion characterized by tremor and marked extensor rigidity of the limbs, especially the fore limbs, and dorsiflexion of the neck with the head ventrally flexed. The convulsive episodes lasted from a few seconds to more than one minute and recovery was accompanied by residual signs of incoordination and tremor. These signs were resolved within two days although no treatment was administered.

The second foal in this group inoculated with virulent ERV (2377) developed respiratory signs similar to those described above, but five days postinoculation, the clinical severity was increased. The rectal temperature at this time was 104°F and marked depression was accompanied by extreme dyspnea, abdominal breathing and flaring of the nostrils. Harsh vesicular lung sounds and cardiac muffling were found on auscultation. Death occurred later that day.

A number of striking lesions were observed on postmortem examination of this animal. The pleural cavity contained approximately three liters of clear yellow fluid with a few strands of fibrin adherent to the visceral and parietal pleura. Both lungs were of uniformly increased density and their dependent areas displayed interlobular and subpleural edema. Similarly, a considerable amount of edema fluid was present in

the mediastinum and the pulmonary airways contained tenaceous frothy mucus with traces of fibrin. The submandibular lymph nodes were congested and focal radial cortical hemorrhages were present in both kidneys. No other gross lesions were observed.

Histologically, the brain, liver, kidney, and adrenal glands of this animal showed evidence of congestion but little inflammatory infiltration. Extensive subpleural, interlobular and alveolar edema and focal areas of atalectasis were present in the lungs. The interalveolar septae were thickened due to congestion and edema, and hypertrophy of the alveolar lining cells, some of which had sloughed into the alveolar lumen, together with smaller mononuclear cells. Mild degenerative changes were present in the tracheal mucosa and involved either ballooning or shrinkage and pyknosis of epithelial cells. The germinal centers of the splenic lymphoid follicles showed evidence of degeneration and the periarterial lymphoid cuffs in the spleen were poorly developed. The bronchial, mesenteric and peripheral lymph nodes all exhibited degrees of congestion, edema, and hemorrhage, with necrosis of germinal centers and increased numbers of active sinus macrophages. Virus isolation and fluorescent antibody studies were not attempted, but the above clinical signs and pathological lesions, along with the detection of eosinophilic intranuclear inclusion bodies in histiocytic cells in the submucosal lymphoid tissues of the small intestine, were taken as sufficient evidence for a diagnosis of death due to virulent ERV infection.

The six foals in group 3 were given daily intramuscular injections of DXM from one-to-nine days of age. Of these, two were intravenously inoculated with virulent ERV at four days of age, two were incoulated with five times and two with three times the recommended dose of an attenuated ERV vaccine.[1] None of the foals inoculated with attenuated vaccine developed increased temperatures or clinical signs of disease during the investigation, except that one animal developed a transient diarrhea between fifteen and seventeen days of age. One of the foals

[1] Rhinomune$^{R}$, Norden Laboratories Inc., Lincoln, Nebraska.

inoculated with virulent ERV became pyretic and slightly depressed one day postinoculation. This pyrexia persisted for six days during which time the rectal temperature fluctuated between 102.2°F and 104°F. A bilateral nasal discharge appeared two days postinoculation and became more profuse and purulent in character before resolution in about one week. During this time, the submandibular lymph nodes were enlarged and a bilateral conjuctivitis and keratitis were accompanied by epiphora. A corneal ulcer developed on one eye and persisted for ten days but this appeared to be due to trauma rather than ERV infection.

The second foal (3077) developed a fever two days after inoculation with virulent ERV, and this persisted up to the time of death five days later. Clinical signs during this time consisted of marked depression, anorexia, conjunctivitis, bilateral mucopurulent nasal discharge, tachypnea and marked hyperpnea. Gross and histological lesions were almost identical to those described above for foal 2377. In addition, very little thymic tissue could be found and the lymph nodes appeared smaller than normal. There were also areas of emphysema in the lungs.

The six foals in group 2 were treated with DXM from one to nine days of age but were not inoculated with ERV. One foal developed a mild diarrhea between five and seven days of age related to the occurrence of estrus in the dam, otherwise no signs of disease were clinically apparent in any of the foals during the period of DXM administration. However, a remarkably consistent pattern of clinical signs developed in all six foals at ten days of age characterized by coughing and mucopurulent nasal discharges. In addition, one foal had an audible tracheal "rattle" and two had enlarged submandibular lymph nodes, one of which abscessed and drained to the intermandibular skin surface. These signs persisted between two and twelve days in different foals, but only one displayed any pyrexia; this being mild (102.6°F) and transient.

It thus seems likely that DXM treatment predisposed these foals to the pathogenic effects of environmental viral and/or bacterial agents

which produced mild clinical signs of disease after cessation of therapy.

## D. Hematologic Values in Foals

Blood samples were drawn from foals in the four treatment groups at one, four, seven, twelve, seventeen, and twenty-two days of age to evaluate the influence of dexamethasone (DXM) treatment and equine rhinopneumonitis virus (ERV) inoculation on several important hematological markers of immunocompetence. These include total white blood cell (wbc), total neutrophil, band neutrophil, lymphocyte, monocyte and eosinophil counts. In addition, it has been shown that plasma fibrinogen concentrations, especially when correlated with total plasma protein levels, are a reasonable indicator of ongoing inflammatory responses in large domestic animals (190). Therefore, these two parameters were measured to assess the effects of DXM and ERV on this essential body response.

It became apparent during the course of this investigation that young pony foals had hematological values which were markedly different from the accepted normal values for adult horses (190). In addition, appreciable changes occurred with increasing age and these tended to override the expected effects of DXM and ERV treatment. Consequently, the hematology results for all four treatment groups were pooled for each sampling day to elucidate these age effects and provide a "baseline" for evaluation of alterations induced by the treatment regimens. In addition, since foals in groups 2 and 3 were given DXM during the first nine days of life, values from four-day-old foals in groups 1 and 4 only were pooled to eliminate any possible interference of DXM during this time. A statistical analysis, employing the analysis of variance procedure was then performed to compare hematology values for all foals at one day of age, before any experimental treatments were given, with those of four-day-old non-DXM-treated foals and foals from all four groups at 22 days of age. These results are shown in Table 13.

The mean total wbc counts rose steadily during the first seventeen

Table 13. Effect of age on hematological parameters in foals

| Parameter | Groups 1 and 4 combined | Groups 1, 2, 3, and 4 combined | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 4 (9)[a] | Day 1 (21)[a] | Day 4 (20)[a] | Day 7 (20)[a] | Day 12 (19)[a] | Day 17 (17)[a] | Day 22 (18)[a] |
| Total wbc[b] | 7001 | 5911[f] | 7829 | 8085 | 9032 | 9972 | 9583[f,****] |
| Total neutrophil[b] | 5372 | 3876[f] | 5926 | 5776 | 6654 | 7368 | 6612[f,***] |
| Band neutrophil[b] | 147[e,****] | 25[e] | 94 | 45 | 81 | 35 | 56 |
| Lymphocyte[b] | 1494[e,*] | 1965[e,f] | 1722 | 2183 | 2257 | 2683 | 2874[f,**] |
| Monocyte[b] | 124 | 51 | 133 | 104 | 92 | 60 | 57 |
| Eosinophil[b] | 0 | 16 | 0 | 13 | 24 | 32 | 21 |
| Plasma fibrinogen[c] | 433[e,**] | 252[e,f] | 315 | 326 | 379 | 470 | 475[f,****] |
| Plasma protein[d] | 6.55 | 6.67 | 6.62 | 6.31 | 5.86 | 6.22 | 6.23 |

[a] Number of animals.

[b] Mean counts/$mm^3$

[c] Mean concentration (mg %).

[d] Mean concentration (g %).

[e] Day 1 (all foals) vs. day 4 (groups 1 and 4 combined).

[f] Day 1 (all foals) vs. day 22 (all foals).

* $p<0.05$.

** $p<0.01$.

*** $p<0.001$.

**** $p<0.0001$.

days of life before values stabilized. Counts at one day of age were about 60% of those found in twenty-two-day-old foals (Figure 10) and the difference between these two age groups was highly significant ($p<0.0001$). The total wbc count changes with increasing age are illustrated for each foal group in Figure 11. The total neutrophil counts (Figure 12) showed a pattern of increase which was essentially identical to the age-related changes observed for total wbc counts and, again, mean values in twenty-two-day-old foals were significantly higher ($p<0.001$) than those found in one-day-old foals (Figure 10).

Lymphocyte counts (Figure 13) fell significantly in untreated foals during the first four days of life ($p<0.05$), but subsequently rose between each sampling interval. This increase was not quite as marked as the increase in neutrophil counts, but was nevertheless statistically significant ($p<0.01$); values at one day of age were about 68% of the mean values in twenty-two-day-old foals (Figure 10).

Band neutrophil, eosinophil, and monocyte counts were generally low and did not show consistent trends during the investigation.

Plasma fibrinogen concentrations (Figure 14) rose very dramatically during the first four days of life in foals not treated with DXM. This trend was masked somewhat by DXM treatment when values for all four groups were combined, but a steady increase in concentrations was still evident up to twenty-two days of age when values were almost double those found in one-day-old foals (Figure 10), a difference which was highly significant ($p<0.0001$).

Plasma protein concentrations did not show significant changes during the period of investigation, although values showed a steady decline during the first twelve days of life before increasing again on days seventeen and twenty-two. This initial decline probably reflected the twice daily collection of blood from the foals during the first twelve days.

Figure 10. A histogram illustrating the effect of age on plasma fibrinogen concentrations, total blood leukocyte counts, total blood neutrophil counts, and blood lymphocyte counts. Values shown are combined means for groups 1, 2, 3 and 4 on the respective days

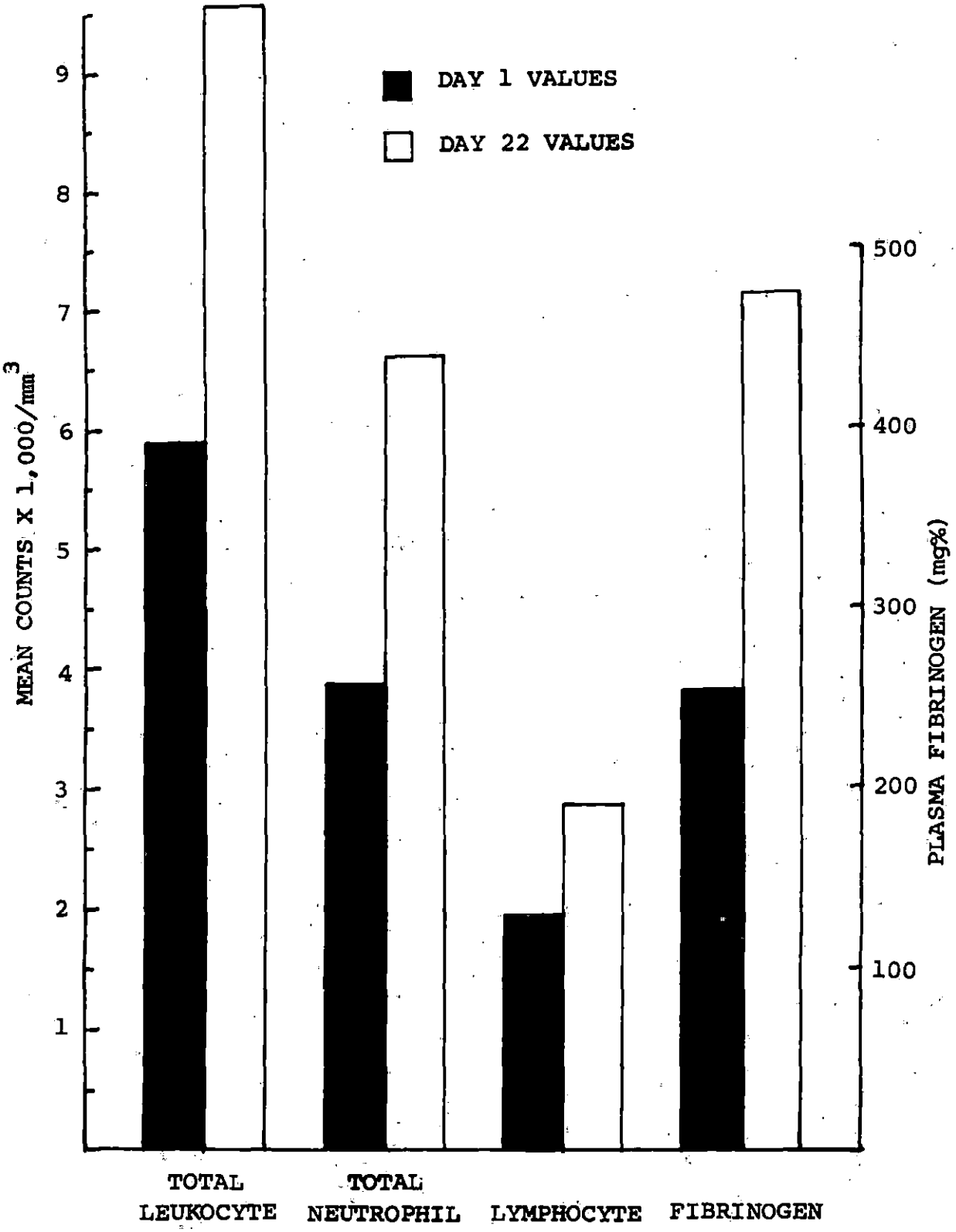

DAY 1 VALUES
DAY 22 VALUES
MEAN COUNTS X 1,000/mm3
PLASMA FIBRINOGEN (mg%)
1
2
3
4
5
6
7
8
9
100
200
300
400
500
TOTAL LEUKOCYTE
TOTAL NEUTROPHIL
LYMPHOCYTE
FIBRINOGEN

Figure 11. A graph illustrating the effects of age, dexamethasone (DXM), and equine rhinopneumonitis virus (ERV) on the total blood leucocyte counts of foals. Animals in groups 2 and 3 received daily intramuscular injections of DXM from one to nine days of age and those in groups 3 and 4 were given ERV intravenously on day four

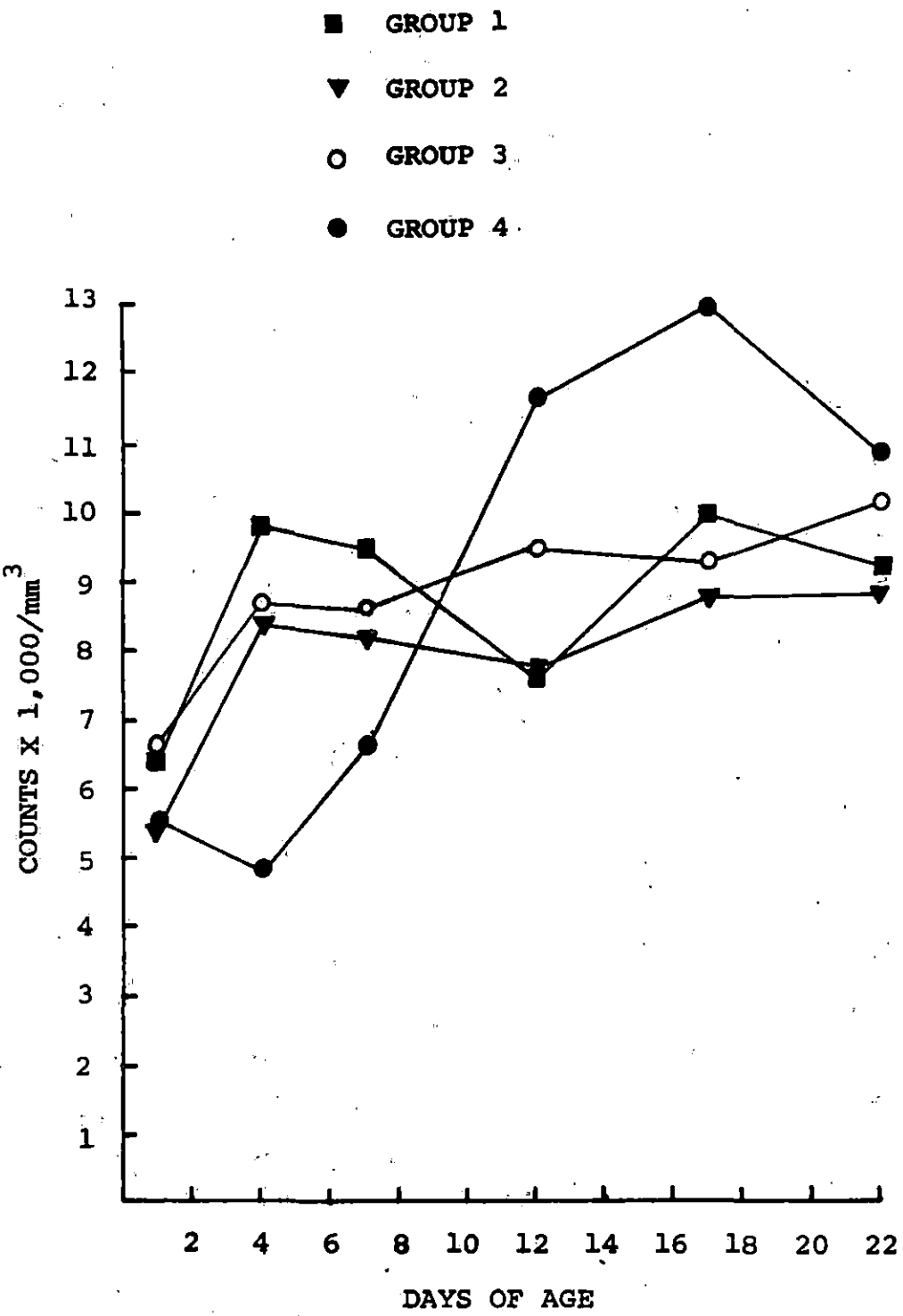

GROUP 1
GROUP 2
GROUP 3
GROUP 4
COUNTS X 1,000/mm3
13
12
11
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
12
14
16
18
20
22
DAYS OF AGE

Figure 12. A graph illustrating the effects of age, dexamethasone (DXM), and equine rhinopneumonitis virus (ERV) on the total blood neutrophil counts of foals. Animals in groups 2 and 3 received daily intramuscular injections of DXM from one to nine days of age and those in groups 3 and 4 were given ERV intravenously on day four

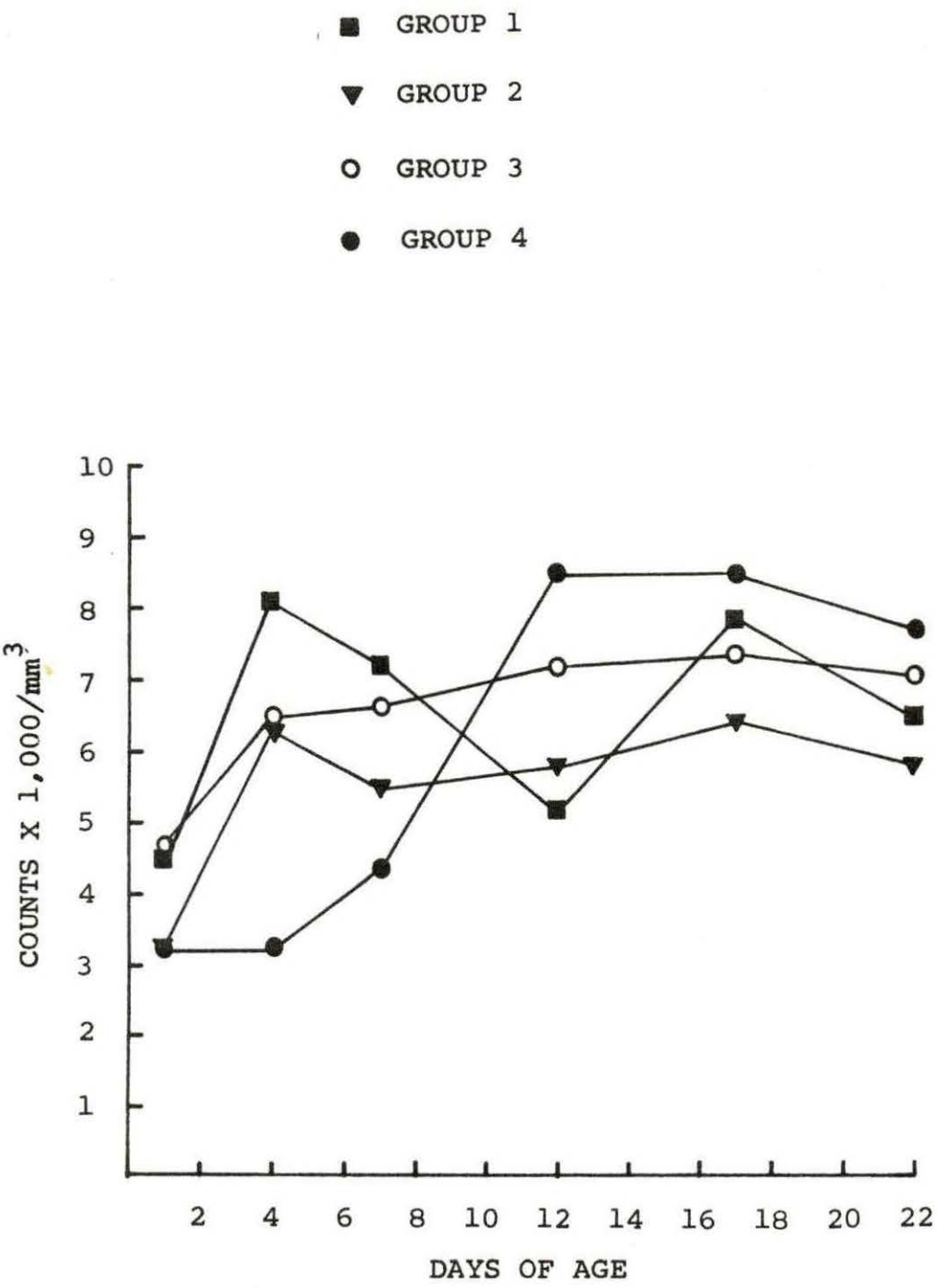

GROUP 1
GROUP 2
GROUP 3
GROUP 4
COUNTS X 1,000/mm3
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
12
14
16
18
20
22
DAYS OF AGE

Figure 13. A graph illustrating the effects of age, dexamethasone (DXM), and equine rhinopneumonitis virus (ERV) on the blood lymphocyte counts of foals. Animals in groups 2 and 3 received daily intramuscular injections of DXM from one to nine days of age, and those in groups 3 and 4 were given ERV intravenously on day four

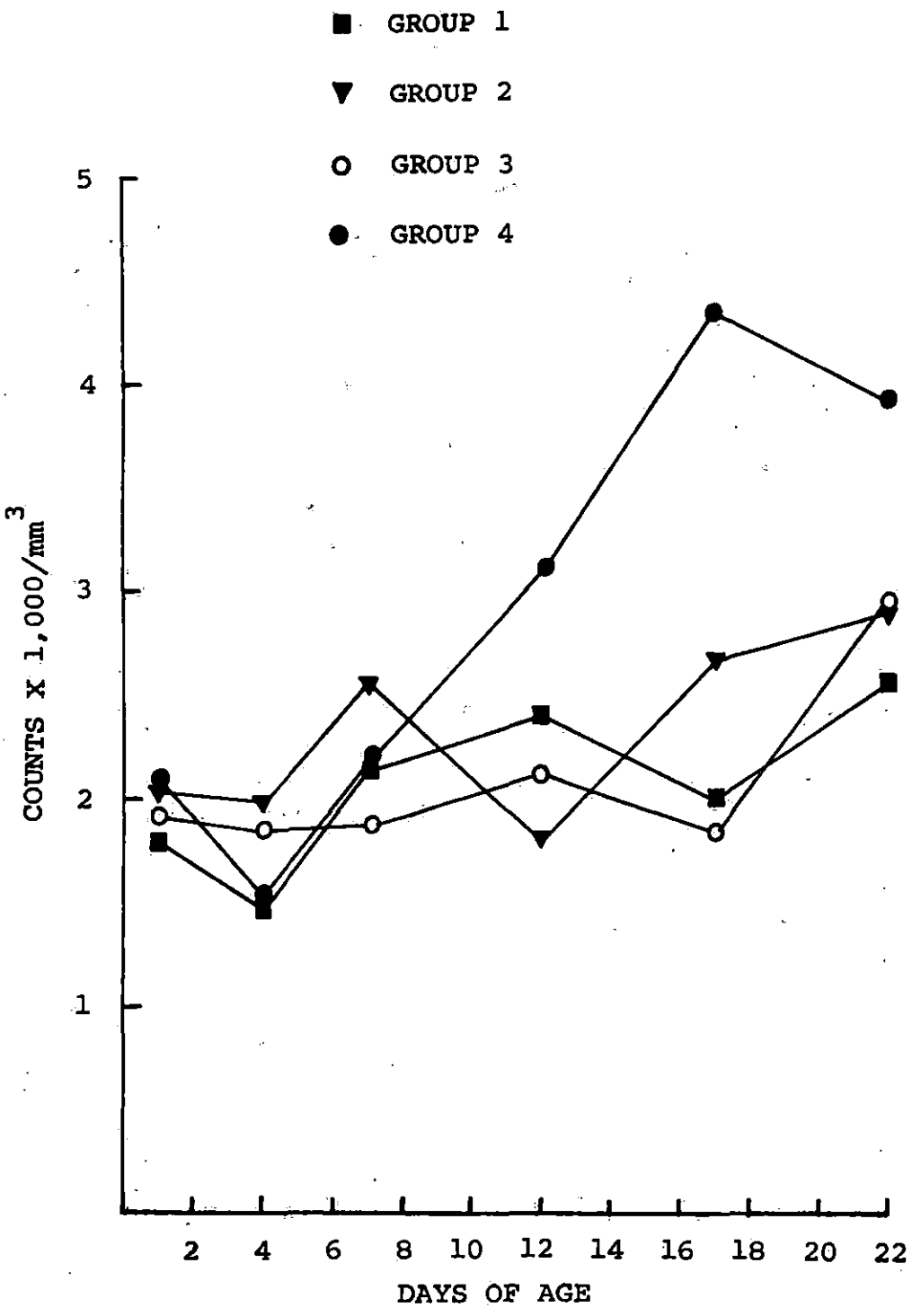
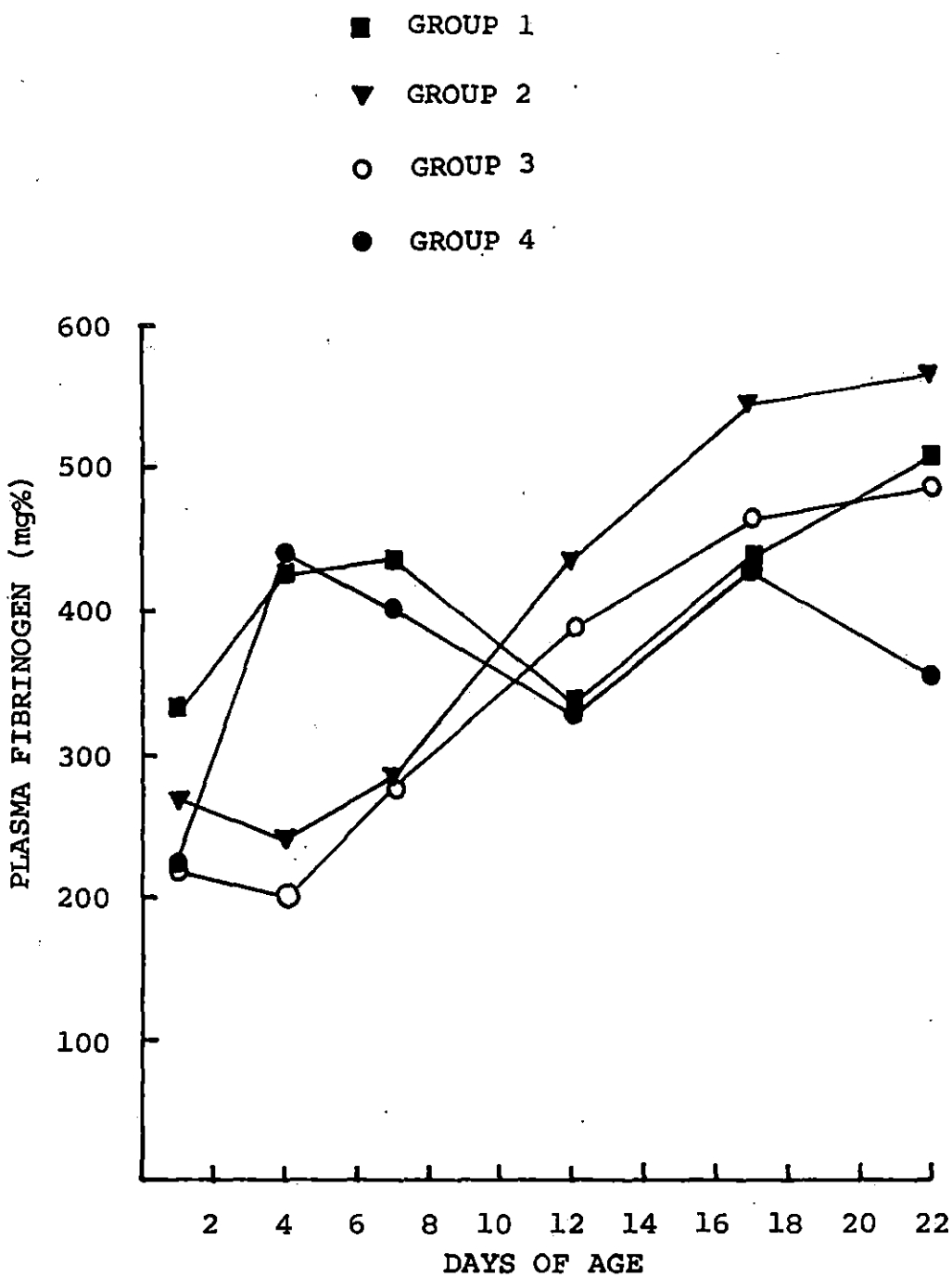

GROUP 1
GROUP 2
GROUP 3
GROUP 4
5
4
3
2
1
COUNTS x 1,000/mm3
2
4
6
8
10
12
14
16
18
20
22
DAYS OF AGE

Figure 14. A graph illustrating the effects of age, dexamethasone (DXM), and equine rhinopneumonitis virus (ERV) on the plasma fibrinogen concentrations of foals. Animals in groups 2 and 3 received daily intramuscular injections of DXM from one to nine days of age and those in groups 3 and 4 were given ERV intravenously on day four


GROUP 1
GROUP 2
GROUP 3
GROUP 4
PLASMA FIBRINOGEN (mg%)
600
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
22
DAYS OF AGE

1. Effect of dexamethasone

The hematology results of the two DXM-treated groups were pooled, as were those of the two nontreated groups, in order to evaluate the effect of DXM treatment on each sampling day (Table 14).

The total wbc, total neutrophil, and lymphocyte counts on the fourth and seventh days of DXM administration were all slightly higher in treated foals, but these trends were subsequently reversed after cessation of therapy. Band neutrophil, monocyte, and eosinophil counts were all low and were not obviously influenced by DXM administration.

Plasma fibrinogen concentration was the only parameter to be significantly influenced by DXM administration. The marked increase in concentrations that occurred in control foals during the first four days of life was not evident in DXM-treated foals and thus values were significantly lower in treated foals than in control foals at both four ($p<0.001$) and seven ($p<0.05$) days of age. After cessation of DXM therapy, plasma fibrinogen concentrations "rebounded" to levels higher than those found in nontreated foals and this difference was found to be significant ($p<0.05$) when values for foals in each group on days twelve, seventeen, and twenty-two were pooled and compared.

2. Effect of equine rhinopneumonitis virus (ERV) treatment

A comparison was made between the hematologic values of foals given ERV at four days of age, and those not treated with ERV, in order to evaluate the effect of such treatment (Table 15).

The total wbc, total neutrophil, and lymphocyte counts were all lower in the ERV-treated foals three days postinoculation, but these trends were subsequently reversed so that all three parameters had higher values in virus-treated animals at eight, thirteen, and eighteen days post-inoculation. These findings were largely a reflection of the pattern of changes which occurred in the virus-treated foals which did not receive DXM (group 4), as illustrated in Figures 10, 11, and 12. Foals in group 4 had mean total neutrophil and lymphocyte counts which were higher than those of all other groups at eight, thirteen, and

Table 14. Effect of dexamethasone (DXM) on hematological values in foals

| Parameter | Treatment status | Age (days) 1 | | 4 | | 7 | | 12 | | 17 | | 22 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total wbc[a] | DXM | 5967 | (12)[b] | 8506 | (11) | 8375 | (12) | 8550 | (12) | 8927 | (10) | 8309 | (11) |
| | No DXM | 5837 | (9) | 7001 | (9) | 7650 | (8) | 9857 | (7) | 11614 | (7) | 10114 | (7) |
| Total neutrophil[a] | DXM | 3932 | (12) | 6379 | (11) | 6038 | (12) | 6440 | (12) | 6827 | (10) | 6307 | (11) |
| | No DXM | 3802 | (9) | 5372 | (9) | 5381 | (8) | 7019 | (7) | 8140 | (7) | 7092 | (7) |
| Band neutrophil[a] | DXM | 28 | (12) | 51* | (11) | 59 | (12) | 79 | (12) | 16 | (10) | 22 | (11) |
| | No DXM | 22 | (9) | 147* | (9) | 25 | (8) | 85 | (7) | 64 | (7) | 111 | (7) |
| Lymphocyte[a] | DXM | 1946 | (12) | 1908 | (11) | 2203 | (12) | 1954 | (12) | 2232 | (10) | 2891 | (11) |
| | No DXM | 1962 | (9) | 1494 | (9) | 2153 | (8) | 2774 | (7) | 3329 | (7) | 2847 | (7) |
| Monocyte[a] | DXM | 47 | (12) | 139 | (11) | 120 | (12) | 110 | (12) | 56 | (10) | 57 | (11) |
| | No DXM | 56 | (9) | 124 | (9) | 80 | (8) | 61 | (7) | 65 | (7) | 57 | (7) |
| Eosinophil[a] | DXM | 17 | (12) | 0 | (11) | 4 | (12) | 34 | (12) | 40 | (12) | 29 | (11) |
| | No DXM | 16 | (9) | 0 | (9) | 28 | (8) | 5 | (7) | 21 | (7) | 9 | (7) |
| Plasma fibrinogen[c] | DXM | 242 | (12) | 218*** | (11) | 275* | (12) | 408 | (12) | 500 | (10) | 520 | (11) |
| | No DXM | 267 | (9) | 433*** | (9) | 414* | (7) | 328 | (7) | 428 | (7) | 400 | (6) |
| Plasma protein[d] | DXM | 6.68 | (12) | 6.67 | (11) | 6.39 | (12) | 5.93 | (12) | 6.37 | (10) | 6.35 | (11) |
| | No DXM | 6.64 | (9) | 6.55 | (9) | 6.15 | (7) | 5.75 | (7) | 6.01 | (7) | 6.02 | (6) |

[a] Mean counts/$mm^3$.

[b] Number of foals.

[c] Mean concentration (mg %).

[d] Mean concentration (g %).

* DXM-treated vs. nontreated foals ($p<0.05$).

*** DXM-treated vs. nontreated foals ($p<0.001$).

Table 15. Effect of equine rhinopneumonitis virus (ERV) treatment on hematologic parameters

| Parameter | Treatment status | Age (days) 1 | 4 | 7 | 12 | 17 | 22 |
|---|---|---|---|---|---|---|---|
| Total WBC[a] | ERV | 6063 (11)[b] | 6897 (11) | 7673 (11) | 10290 (10) | 10844 (9) | 10344 (9) |
| | No ERV | 5743 (10) | 8968 (9) | 8589 (9) | 7366 (9) | 9100 (8) | 8822 (9) |
| Total neutrophil[a] | ERV | 4000 (11) | 4993 (11) | 5566 (11) | 7652 (10) | 7790 (9) | 7258 (9) |
| | No ERV | 3741 (10) | 7066 (9) | 6031 (9) | 5545 (9) | 6894 (8) | 5968 (9) |
| Band neutrophil[a] | ERV | 9 (11) | 77 (11) | 35 (11) | 56 (10) | 29 (9) | 83 (9) |
| | No ERV | 43 (10) | 109 (9) | 59 (9) | 110 (9) | 43 (8) | 30 (9) |
| Lymphocyte[a] | ERV | 1995 (11) | 1695 (11) | 1996 (11) | 2498 (10) | 2937 (9) | 2994 (9) |
| | No ERV | 1932 (10) | 1755 (9) | 2413 (9) | 1989 (9) | 2399 (8) | 2753 (9) |
| Monocyte[a] | ERV | 48 (11) | 118 (11) | 85 (11) | 89 (10) | 30*(9) | 58 (9) |
| | No ERV | 53 (10) | 151 (9) | 127 (9) | 89 (9) | 93*(8) | 56 (9) |
| Eosinophil[a] | ERV | 21 (11) | 0 (11) | 10 (11) | 32 (10) | 50 (9) | 13 (9) |
| | No ERV | 11 (10) | 0 (9) | 17 (9) | 14 (9) | 11 (8) | 30 (9) |
| Plasma fibrinogen[c] | ERV | 218 (11) | 309 (11) | 320 (10) | 367 (10) | 444 (9) | 422 (9) |
| | No ERV | 290 (10) | 322 (9) | 333 (9) | 400 (9) | 500 (8) | 414 (7) |
| Plasma protein[d] | ERV | 6.95 (11) | 6.85 (11) | 6.42 (10) | 5.96 (10) | 6.35 (9) | 6.59** (9) |
| | No ERV | 6.36 (10) | 6.33 (9) | 6.18 (9) | 5.76 (9) | 6.07 (8) | 5.76** (7) |

[a] Mean counts/$mm^3$.

[b] Number of foals.

[c] Mean concentration (mg%).

[d] Mean concentration (g %).

* ERV inoculated vs. noninoculated foals ($p<0.05$).

** ERV inoculated vs. noninoculated foals ($p<0.01$).

eighteen days postinoculation, and these changes were magnified when the total wbc counts were examined over the same period (Figure 10).

No marked effect of ERV treatment was apparent when band neutrophil, eosinophil, and monocyte counts, and plasma fibrinogen concentrations in ERV inoculated foals and noninoculated foals were compared. Inoculated foals did, however, have higher plasma protein concentrations, although these results may not represent a true effect of ERV inoculation in that the noninoculated group had appreciably lower values even before administration of the virus.

## E. Humoral Response to Antigens

The inoculation and vaccination procedures, and subsequent serological evaluations, employed in this investigation permitted an assessment of the ability of young foals to mount humoral responses to three distinct antigens and the influence of pre-existing colostral antibodies on these responses. Furthermore, the daily administration of DXM to half of the foals (groups 2 and 3), during the first nine days of life, made possible an evaluation of both the short- and long-term effects of this potent glucocorticosteroid on humoral immune function.

Foals in groups 3 and 4 were given equine rhinopneumonitis virus (ERV) intravenously at four days of age, and animals in all four groups received vaccinal ERV intramuscularly when about forty days old. Serum samples were drawn during this period to measure ERV neutralizing antibody titers.

Foals in all four groups had demonstrable anti-ERV antibody titers of colostral origin at one day of age and, since these varied widely between individuals, the logarithm to the base two ($\log_2$) of the titers were calculated to aid in the interpretation of results. These results are illustrated graphically by group in Figure 15.

Foals inoculated with ERV at four days of age showed a decline in anti-ERV titers during the first twelve days of life and similar trends were evident in noninoculated foals (Table 16). This titer decline continued in the noninoculated group, whereas mean titers increased in the

Figure 15. A graph illustrating the effects of dexamethasone (DXM) and equine rhinopneumonitis virus (ERV) on the anti-ERV antibody titers of foals. Animals in groups 2 and 3 were given daily intramuscular injections of DXM from one to nine days of age. ERV was administered intravenously to foals in groups 3 and 4 at four days of age and all foals were later vaccinated with a modified live ERV vaccine

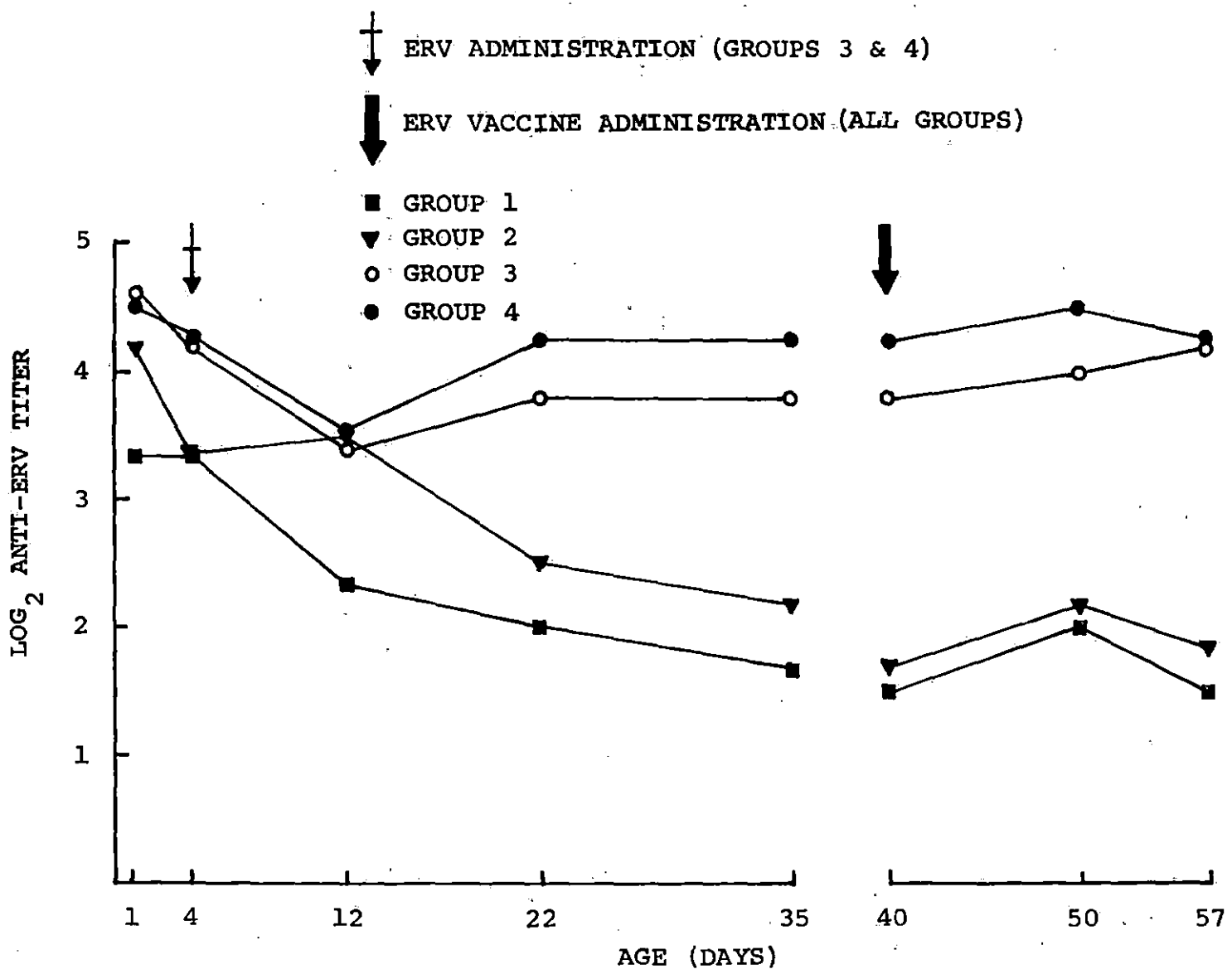

ERV ADMINISTRATION (GROUPS 3 & 4)
ERV VACCINE ADMINISTRATION (ALL GROUPS)
GROUP 1
GROUP 2
GROUP 3
GROUP 4
LOG2 ANTI-ERV TITER
5
4
3
2
1
1
4
12
22
35
40
50
57
AGE (DAYS)

Table 16. Effect of equine rhinopneumonitis virus (ERV) inoculation on neutralizing antibody titers

| Age (days) | $Log_2$ ERV-neutralizing antibody titer[a] | | | |
|---|---|---|---|---|
| | ERV-treated | | Nontreated | |
| 1 | 4.56 ± .38 (9)[b] | | 3.89 ± .39 (9) | |
| 4[c] | 4.22 ± .36 (9) | | 3.33 ± .44 (9) | |
| 12 | 3.44 ± .38 (9) | | 3.11 ± .42 (9) | |
| 22 | 4.00 ± .41 (9) | 3.81****(27) | 2.33 ± .41 (9) | 2.86****(26) |
| 35 | 4.00 ± .75 (9) | | 2.00 ± .41 (8) | |
| 40[d] | 4.00 ± .75 (9) | | 1.67 ± .44 (8) | |
| 50 | 4.22 ± .66 (9) | 4.22****(18) | 2.13 ± .30 (8) | 1.94****(16) |
| 57 | 4.22 ± .60 (9) | | 1.75 ± .25 (8) | |

[a]Mean ± standard error of the mean.

[b]Number of foals.

[c]ERV inoculation.

[d]ERV vaccine administration.

****ERV-treated vs. nontreated ($p<0.0001$).

inoculated group by eighteen days after ERV administration and remained elevated seventeen days later. In order to further evaluate this antibody response, anti-ERV titers on the eighth, eighteenth, and thirty-first postinoculation days were pooled and compared with titers in non-inoculated foals of equivalent age. Titers were found to be significantly higher ($p<0.0001$) in the ERV-inoculated group indicating that a primary humoral response to the virus had occurred.

All foals were vaccinated with an attenuated ERV product[1] at about forty days of age. A serologic response was noted in control foals as well as those previously inoculated with ERV. However, postvaccinal titers were significantly higher ($p<0.0001$), and the rise more sustained, in the latter group indicating an anamnestic response took place in

[1]Rhinomune$^R$, Norden Laboratories Inc., Lincoln, Nebraska.

foals already sensitized to the virus (Table 16).

Neither the development of serological responses to ERV, nor the disappearance of antibodies from the circulation, appeared to be influenced by DXM treatment.

Brucella abortus bacterin was administered subcutaneously to all foals at three days of age to further evaluate the effect of DXM on humoral immune functions. Again, many foals had significant anti-brucella agglutinating antibody titers of maternal origin at one day of age and these varied considerably in magnitude between individuals. The serologic responses were thus expressed in terms of $\log_2$ antibody titers.

When the responses of DXM-treated and non-DXM-treated foals were compared (Table 17), it became obvious that DXM treatment at the time of vaccination did not influence the agglutinin response of foals to Brucella abortus. Both groups developed increased levels of circulating anti-brucella antibody within eight days of vaccination, with a further titer rise peaking five days later.

Table 17. Effect of dexamethasone (DXM) on the serological response of foals to Brucella abortus bacterin

| Age (days) | $\log_2$ anti-Brucella agglutinating antibody titer[a] | |
|---|---|---|
| | DXM-treated foals | Nontreated foals |
| 1 | 6.23 ± .72 (11)[b] | 5.61 ± .84 (7) |
| 3[c] | 5.38 ± .88 (11) | 4.42 ± 1.04 (7) |
| 4 | 4.45 ± .94 (11) | 2.90 ± 1.16 (7) |
| 12 | 5.41 ± .41 (11) | 4.13 ± .84 (7) |
| 17 | 7.32 ± .47 (11) | 6.75 ± .43 (7) |
| 22 | 6.50 ± .62 (11) | 6.75 ± .37 (7) |

[a] Mean ± standard error of the mean.

[b] Number of animals.

[c] Brucella abortus bacterin inoculation.

Sheep red blood cell (SRBC) antigen was intravenously administered to foals at twelve days of age. None of the animals showed preinoculation titers of more than two, and all developed a positive serologic response. As can be seen from Table 18, titers peaked ten days after the administration of the SRBC's and this response was unaffected by DXM treatment.

Table 18. Effect of dexamethasone (DXM) on the serological response to sheep red blood cell (SRBC) antigen

| Age (days) | $Log_2$ anti-SRBC hemagglutinating antibody titer[a] | |
|---|---|---|
| | DXM-treated | Nontreated |
| 12[b] | .88 ± .13 (8)[c] | .40 ± .24 (5) |
| 22 | 3.75 ± .25 (8) | 3.80 ± .37 (5) |
| 35 | 2.75 ± .31 (8) | 2.80 ± .20 (5) |

[a]Mean ± standard error of the mean.

[b]SRBC inoculation.

[c]Number of animals.

## F. *In Vitro* Blastogenic Responses of Lymphocytes

The *in vitro* blastogenic responses of lymphocytes to antigens and certain plant mitogens are frequently used to monitor cell-mediated immune (CMI) functions, dependent on T-lymphocytes, in the horse and other species (11,65,78,85,158,209,226). Since there are few previous reports concerning the assessment of this essential body defense mechanism in very young foals, it was decided to investigate this process with specific reference to age-related effects and the ability of ERV to stimulate T-lymphocytes to mount a specific CMI response. In addition, the influence of glucocorticosteroids on CMI responses was investigated by administering DXM to half the foals (groups 2 and 3) during the first

nine days of life.

Lymphocytes were collected from foals in the four groups at four, twelve, and seventeen days of age and cultured in vitro with phytohemagglutinin (PHA), concanavalin A (Con A), pokeweed mitogen (PWM), ERV, and rabbit anti-equine globulin (AG). The latter is thought to stimulate predominantly B-lymphocytes (50) and was used to help investigate the B-cell component of the immune system, in conjunction with the specific antibody responses already reported.

The blastogenic responses of lymphocytes cultured with the above agents were expressed in terms of the net counts per minute (net cpm), the stimulation index (S.I.), and the logarithm to the base ten of the stimulation index ($\log_{10}$ S.I.), to enable a meaningful analysis to be carried out.

Lymphocytes from four-day-old foals showed appreciable blastogenic responses to stimulation with PHA, Con A, and PWM. Although there was considerable variation between individual animals, mean responses were equivalent to those of twelve and seventeen-day-old foals and adult pony mares studied concurrently, indicating that this aspect of CMI function is highly developed from a very early age in foals.

The anti-globulin responses were less well-developed in four-day-old foals, but by the time these foals were twelve and seventeen days of age, responses were found to be of similar magnitude to those seen in adult pony mares.

DXM treatment had no appreciable effect on in vitro blastogenic responses to PHA but significant reductions were observed in the response to Con A ($p<0.01$), PWM ($p<0.01$), and AG ($p<0.05$), when these were expressed in terms of Net cpm (Table 19).

When S.I. and $\log_{10}$ S.I. were used as criteria for assessment of in vitro lymphocyte blastogenesis, only the AG responses were found to be significantly reduced by DXM treatment ($p<0.05$).

At twelve days of age, i.e., three days after the last DXM treatment, lymphocyte responses to PHA were significantly higher in the treated group than in nontreated controls ($p<0.05$). In addition, blastogenic

responses to Con A and PWM were also increased in previously treated foals at this time. These findings may indicate that a population of mitogen responsive, steroid resistant, T-lymphocytes was selected during the period of DXM treatment and that these cells proliferated after withdrawal of the drug. This effect was not, however, apparent when foals reached seventeen days of age, at which time animals in both treatment groups had equivalent responses to all three mitogens (Table 19).

The blastogenic responses of lymphocytes to AG, at twelve and seventeen days of age, were still lower in DXM-treated foals than in control foals; this effect being statistically significant ($p<0.05$) at seventeen days of age when stimulation indices and $\log_{10}$ S.I. were considered.

The above results (Tables 19 and 20) indicate that DXM induces a much more marked and prolonged suppressive effect on B-cell responses than on T-cell responses, suggesting that, in the intact foal, CMI mechanisms should be minimally affected, whereas humoral responses to antigens may be inhibited by DXM therapy.

1. Effect of equine rhinopneumonitis virus

Half of the foals in this investigation were inoculated intravenously with ERV at four days of age (groups 3 and 4), while the remaining foals served as controls. The comparative *in vitro* responses of lymphocytes from inoculated and noninoculated foals are shown in Tables 21 and 22, from which it can be seen that, eight days post-inoculation, the blastogenic responses to PHA, Con A, and PWM, were all significantly depressed in ERV-treated foals when net cpm was used as the criterion for blastogenesis ($p<0.05$). This apparent ERV-induced inhibition of CMI responses was not evident when S.I. and $\log_{10}$ S.I. were used to assess blastogenesis. The reason for these rather confusing results is not known, but such an effect could be observed if *in vitro* blastogenesis in the unstimulated lymphocytes (background counts) was inhibited to the same degree as blastogenesis in ERV-stimulated cells.

Table 19. Effect of dexamethasone (DXM) on lymphocyte stimulation induced by mitogens

| Parameter | Day | MITOGEN: Phytohemagglutinin, DXM[a] | MITOGEN: Phytohemagglutinin, No DXM |
|---|---|---|---|
| Net counts per minute | 4 | 22493±12735[b] (12)[c] | 23770± 5398 (8) |
| | 12 | 58237±13199* (11) | 17972± 5505* (6) |
| | 17 | 30569± 4392 (12) | 53550±20073 (7) |
| | 4+12 | 39588± 9730 (23) | 21285± 3820 (14) |
| Stimulation index | 4 | 56.2± 27.8 (12) | 18.2± 3.0 (8) |
| | 12 | 49.5± 13.2 (11) | 26.8± 10.6 (6) |
| | 17 | 35.0± 8.8 (12) | 30.7± 10.6 (7) |
| | 4+12 | 53.0± 15.5 (23) | 21.9± 4.8 (14) |
| Log stimulation index | 4 | 2.61± .56 (12) | 2.77± .21 (8) |
| | 12 | 3.35± .38 (11) | 2.71± .53 (6) |
| | 17 | 3.11± .30 (12) | 3.16± .28 (7) |
| | 4+12 | 2.96± .34 (23) | 2.74± .25 (14) |

[a]Animals in the treated groups received daily intramuscular injections of DXM from day 1 to day 9.

[b]Values shown are means ± standard errors of the means.

[c]Number of animals.

*DXM-treated vs. nontreated foals ($p<0.05$).

**DXM-treated vs. nontreated foals ($p<0.01$).

| MITOGEN | | | | | | | |
|---|---|---|---|---|---|---|---|
| Concanavalin A | | | | Pokeweed Mitogen | | | |
| DXM[c] | | No DXM | | DXM[c] | | No DXM | |
| 21045± 9462** | (12) | 73583±14270** | (8) | 10797± 5054* | (9) | 51207±10129* | (8) |
| 119380±22067 | (11) | 58011±13813 | (6) | 107106±23927 | (11) | 44267±10878 | (6) |
| 79944±11210 | (12) | 93989±31856 | (7) | 66168±10647 | (12) | 59537±16706 | (6) |
| 68074±15456 | (23) | 66909± 9934 | (14) | 63767±17070 | (20) | 48233± 7210 | (14) |
| 47.4± 16.9 | (12) | 65.6± 15.9 | (8) | 48.0± 22.9 | (9) | 41.1± 9.3 | (8) |
| 111.2± 25.0 | (11) | 101.0± 31.2 | (6) | 82.2± 15.4 | (11) | 82.4± 32.7 | (6) |
| 97.9± 30.1 | (12) | 52.4± 15.7 | (7) | 69.9± 20.5 | (12) | 40.0± 15.8 | (6) |
| 77.9± 16.0 | (23) | 80.8± 16.2 | (14) | 66.8± 13.5 | (20) | 58.8± 15.4 | (14) |
| 2.73± .55 | (12) | 3.95± .26 | (8) | 2.48± .70 | (9) | 3.53± .23 | (8) |
| 4.29± .34 | (11) | 2.71± .54 | (6) | 4.10± .29 | (11) | 3.65± .73 | (6) |
| 3.97± .36 | (12) | 3.75± .25 | (7) | 3.65± .36 | (12) | 3.32± .39 | (6) |
| 3.48± .36 | (23) | 4.05± .25 | (14) | 3.37± .39 | (20) | 3.58± .32 | (14) |

Table 20. Effect of dexamethasone (DXM) on lymphocyte stimulation induced by anti-globulin and equine rhinopneumonitis virus

| Parameter | Day | Anti-globulin | | | | Equine rhinopneumonitis virus | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | DXM[a] | | No DXM | | DXM[a] | | No DXM | |
| Net counts per minute | 4 | 77± 69[b,*] | (7)[c] | 2818±1378* | (5) | -12.6± 145 | (5) | 926 | (1) |
| | 12 | 3626±1769 | (6) | 2980±1136 | (5) | -1402±3206 | (9) | 3821±2485 | (6) |
| | 17 | 4409±2022 | (6) | 8393±2255 | (5) | 2285±1209 | (11) | 1410± 765 | (7) |
| | 4+12 | 1715± 929 | (13) | 2899± 828 | (10) | -905±2026 | (14) | 3408±2141 | (7) |
| Stimulation index | 4 | 1.22± .24* | (7) | 2.37± .34* | (5) | 1.27± .22 | (5) | 1.49 | (1) |
| | 12 | 2.12± .40 | (6) | 10.20±5.12 | (5) | 1.91± .65 | (9) | 2.24± .77 | (6) |
| | 17 | 2.23± .47* | (6) | 4.74±1.01* | (5) | 1.79± .29 | (11) | 1.51± .44 | (7) |
| | 4+12 | 1.63± .25 | (13) | 6.29±2.75 | (10) | 1.68± .42 | (14) | 2.13± .66 | (7) |
| Log stimulation index | 4 | .11± .24* | (7) | .81± .17* | (5) | .18± .18 | (5) | .40 | (1) |
| | 12 | .64± .23 | (6) | 1.69± .61 | (5) | .24± .32 | (9) | .50± .37 | (6) |
| | 17 | .67± .25* | (6) | 1.46± .23* | (5) | .47± .15 | (11) | .23± .22 | (7) |
| | 4+12 | .35± .15* | (13) | 1.25± .33* | (10) | .22± .21 | (14) | .48 ± .31 | (7) |

[a]Treated animals received daily intramuscular injections of DXM from day 1 to day 9.

[b]Values shown are means ± standard errors of the means.

[c]Number of animals evaluated on each day.

*DXM-treated vs. nontreated foals ($p<0.05$).

Table 21. Effect of equine rhinopneumonitis virus (ERV) on lymphocyte stimulation induced by mitogens

| Parameter | Day | MITOGEN: Phatohemagglutinin — Virus[a] | | | MITOGEN: Phatohemagglutinin — No virus | | |
|---|---|---|---|---|---|---|---|
| Net counts | 4 | 30616±13470[b,*] | (11)[c] | | 13700± 4823 | (9) | |
| | 12 | 23103± 7221* | (9) | | 67563±15947* | (8) | |
| | 17 | 48305±14807 | (10) | | 28738± 1899 | (9) | |
| | 12+17 | 36367± 8801 | (19) | | 47009± 8763 | (17) | |
| Stimulation index | 4 | 58.9± 30.1 | (11) | | 19.3± 5.8 | (9) | |
| | 12 | 45.4± 16.6 | (9) | | 37.0± 8.8 | (8) | |
| | 17 | 34.7± 9.7 | (10) | | 32.0± 9.4 | (9) | |
| | 12+17 | 39.8± 9.2 | (19) | | 34.4± 6.3 | (17) | |
| Log stimulation index | 4 | 3.11± .39 | (11) | | 2.14± .55 | (9) | |
| | 12 | 2.98± .51 | (9) | | 3.28± .35 | (8) | |
| | 17 | 3.18± .29 | (10) | | 3.07± .33 | (9) | |
| | 12+17 | 3.09± .28 | (19) | | 3.17± .28 | (17) | |

[a]Animals in the treated groups were given ERV intravenously on day 4.

[b]Values shown are means ± standard errors of the means.

[c]Number of animals.

*ERV-inoculated vs. noninoculated foals ($p<0.05$).

| MITOGEN | | | |
|---|---|---|---|
| Concanavalin A | | Pokeweed mitogen | |
| Virus[a] | No virus | Virus[c] | No virus |
| 51254±15533 (11) | 30822±10451 (9) | 32620± 9804 (10) | 25803±11608 (7) |
| 65479±17537* (9) | 133992±23862* (8) | 53389±14526* (9) | 120408±29168* (8) |
| 93554±24361 (10) | 75747± 8474 (9) | 67596±16615 (9) | 60320± 6869 (9) |
| 80255±15226 (19) | 103156±13750 (17) | 60493±10843 (18) | 88597±15616 (17) |
| 58.1± 18.7 (11) | 50.5± 14.4 (9) | 43.9± 19.6 (10) | 45.9± 14.1 (7) |
| 128.0± 29.9 (9) | 84.7± 21.5 (8) | 99.7± 22.9 (9) | 62.6± 16.2 (8) |
| 62.3± 16.0 (10) | 102.0± 39.9 (9) | 49.7± 17.9 (9) | 70.0± 24.1 (9) |
| 93.4± 17.8 (19) | 93.9± 22.4 (17) | 74.7± 15.4 (18) | 66.6± 14.4 (17) |
| 3.35± .43 (11) | 3.05± .66 (9) | 3.02± .43 (10) | 2.91± .79 (7) |
| 4.46± .37 (9) | 4.03± .40 (8) | 4.05± .52 (9) | 3.82± .33 (8) |
| 3.81± .27 (10) | 3.98± .42 (8) | 3.34± .40 (9) | 3.73± .38 (9) |
| 4.12± .23 (19) | 4.00± .28 (17) | 3.70± .33 (18) | 3.78± .25 (17) |

Table 22. Effect of equine rhinopneumonitis virus (ERV) on the *in vitro* stimulation of lymphocytes by anti-globulin and ERV

| Parameter | Day | Anti-globulin | | | | Equine rhinopneumonitis virus | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Virus[a] | | No virus | | Virus[a] | | No virus | |
| Net counts | 4 | 1792±1091[b] | (7)[c] | 417± 347 | (5) | 160± 82 | (8) | 128± 431 | (3) |
| | 12 | 2393±1133 | (6) | 4459±1879 | (5) | 4074±1885 | (9) | -4393±4122 | (6) |
| | 17 | 5733±2534 | (6) | 6804±1897 | (5) | 3125±1169 | (10) | 470± 785 | (8) |
| | 12+17 | 4062±1416 | (12) | 5632±1318 | (10) | 3574±1058* | (19) | -1614±1853* | (14) |
| Stimulation index | 4 | 1.87± .39 | (7) | 1.45± .28 | (5) | 1.54± .22 | (3) | 1.07± .24 | (3) |
| | 12 | 8.13±4.59 | (6) | 2.99± .44 | (5) | 2.59± .73 | (9) | 1.21± .37 | (6) |
| | 17 | 2.28± .54 | (6) | 4.68± .99 | (5) | 1.98± .37 | (10) | 1.32± .24 | (8) |
| | 12+17 | 5.20±2.37 | (12) | 3.84± .58 | (10) | 2.27± .39 | (19) | 1.27± .20 | (14) |
| Log stimulation index | 4 | .47± .24 | (7) | .31± .17 | (5) | .41± .14 | (3) | .02± .24 | (3) |
| | 12 | 1.17± .62 | (6) | 1.15± .15 | (5) | .62± .30 | (9) | -0.08± .36 | (6) |
| | 17 | .67± .26* | (6) | 1.45± .21* | (5) | .54± .17 | (10) | .18± .16 | (8) |
| | 12+17 | .92± .32 | (12) | 1.25± .14 | (10) | .58± .16* | (19) | .07± .17* | (14) |

[a]Treated animals were given ERV intravenously on day 4.

[b]Values shown are means ± standard errors of the means.

[c]Number of animals.

*ERV-inoculated *vs.* noninoculated foals ($p<0.05$).

Foals inoculated with ERV had better *in vitro* lymphocyte responses to ERV than noninoculated foals on the eighth and thirteenth days after the virus was administered. When data from these two days were pooled, it was found that both the net cpm and $\log_{10}$ S.I. were significantly higher in the ERV-treated group ($p<0.05$). These findings thus provide good evidence that four-day-old foals are capable of mounting CMI responses to challenge with ERV and that this virus is, indeed, able to sensitize the T-lymphocyte system of the equine.

## G. Peripheral Lymph Node Histology

Histological evaluation of lymph node biopsies, collected from all foals at about fourteen days of age, failed to demonstrate any effect of dexamethasone or equine rhinopneumonitis virus treatments on peripheral lymph node histology.

# V. DISCUSSION

## A. Plasma Cortisol Concentrations

Cortisol is the major glucocorticosteroid present in the plasma of horses (28,105,119,229), cattle (176,185,219), sheep (15,45,185,207), pigs (28), dogs (85,90,111), man (95,113,120), monkeys (147) and guinea pigs (52). In these species, measurement of resting plasma cortisol concentrations can be a reasonable guide as to the normality of adrenocortical function, especially if adrenal stimulation and suppression tests are also employed.

The mean daily plasma cortisol concentrations of resting adult ponies and horses in this study were 2.75 $\pm$ .18 µg/dl and 3.00 $\pm$ .18 µg/dl, respectively, and were not significantly different from each other. Both groups exhibited a rather large interindividual and day-to-day individual variation in cortisol levels; pony values ranged from .9 µg/dl to 7.0 µg/dl while horses showed a range of values from .5 µg/dl to 8.4 µg/dl. Hoffsis et al. (97) and James et al. (105) have reported similar day-to-day and interindividual variability.

Kumar et al. (124) performed the only published study concerning the use of radioimmunoassays (RIA) to measure cortisol values in ponies and found mean daily plasma cortisol concentrations of 2.55 µg/dl, which are in very close agreement with those observed in the present study. Mean daily values of 1.37 µg/dl and 1.59 µg/dl have been recorded in adult ponies using a competitive protein binding (CPB) assay (28, 160), and Hoffsis et al. (97) reported levels of 5.1 µg/dl in adult horses of various breeds using a similar procedure. James et al. (105) used a fluorimetric technique to derive mean daily plasma cortisol concentrations of 6.1 µg/dl in adult horses and ponies, with no significant difference between the two. The range of values reported in different studies likely reflects the differences between the assay procedures used. RIA techniques are considered to be the most specific for cortisol with low cross-reactivity from other steroids (75,111) and

thus values derived by this method are generally lower and give a more valid reflection of circulating cortisol concentrations than do those derived by other methods.

Adult ponies and horses in the present study showed a distinct diurnal pattern of variation in plasma cortisol concentrations, with morning samples having significantly higher levels than afternoon samples. Afternoon values were approximately 63% of morning values in ponies and 50% of morning values in horses. Similar diurnal trends have been reported previously in ponies (28,105,124) and horses (97, 105,229). Kumar et al. (124) sampled ponies at two hour intervals and found the peak cortisol concentrations occurred between 9 am and 11 am (3.17 µg/dl) with the lowest levels twelve hours later (1.96 µg/dl), when the animals were maintained on a strict twelve hour, 6 am to 6 pm, lighting regimen. Bottoms et al. (28) used a similar sampling schedule in pony mares and found that the diurnal rhythm was sinusoidal in character; cortisol levels rose during the night to peak at 8 am (2.8 µg/dl), followed by a progressive decline during the day to reach a nadir at 10 pm (.5 µg/dl). Hoffsis et al. (97) observed a similar trend in horses sampled at twenty-eight hour intervals to eliminate the possible disruption caused by collecting the previous sample. Peak concentrations were found at 8 am (4.2 µg/dl) followed by a progressive decline during the day to reach minimum levels at 4 pm (1.7 µg/dl). The diurnal rhythm reported by Zolovick et al. (229) in adult horses was characterized by having low values at 10 pm which were 65% of the peak daily values recorded at 10 am.

In the current investigation, plasma samples were drawn between 7:45 am and 8 am and again between 5 pm and 5:30 pm. It seems likely, in light of the results of the above studies, that these sampling times closely approximated the expected occurrence of maximum and minimum plasma cortisol concentrations.

The mechanism governing the diurnal rhythm of cortisol levels in the horse has not been investigated. In man, the central nervous system appears to impose a diurnal cyclic pattern on the release of

corticotrophin releasing factor (CRF) from the medial eminence of the hypothalamus. This, in turn, stimulates a morning surge of ACTH release from the anterior pituitary and subsequent increased adrenocortical production and release of cortisol (173). Orth et al. (166) showed that, in man, this rhythm is related to the habitual sleep-wake cycle and can be altered by changing the sleeping habits for several days but not by abrupt changes. In contrast, the diurnal pattern in sheep seems to be directly governed by the dark-light cycle since if sheep are acutely exposed to light for an entire twenty-four hours, the diurnal rhythm is lost (137).

The plasma cortisol concentrations of foals were measured, beginning at twelve to twenty-four hours of age and continuing throughout the first twenty-two days of life. Mean cortisol levels on the first day (2.49 µg/dl) were slightly lower than those of adult ponies (2.74 µg/dl) and declining values over the next two or three days resulted in mean daily concentrations in foals two-to-four days of age (1.89 µg/dl) that were significantly less than those of adults. Levels subsequently rose and, in spite of day-to-day fluctuations, were equivalent to adult concentrations at one week of age and were slightly higher than adult values between twelve and twenty-two days of age.

The literature contains no reports regarding the plasma cortisol concentrations in foals during the time period investigated herein. Rossdale et al. (182), however, observed a significant increase in the cortisol levels of thoroughbred foals, from 6.8 µg/dl to 8.3 µg/dl, during the first thirty minutes of life. Levels fell precipitously after three hours and at twelve to twenty-four hours of age averaged 2.4 µg/dl, a value which correlates very well with the concentrations found in foals of similar age in this investigation (2.47 µg/dl). A similar trend has been observed in calves (47,68,101), and lambs (16,62,159, 207) during the first twenty-four hours of life and levels continue to decline up to twelve days of age in cattle (68,101). Total plasma GCS concentrations in newborn infants are approximately three times higher than those of adults. A marked decline during the first forty-

eight hours is followed by a more gradual fall over the next two weeks of life but adult values are not attained until one-to-five months of age (120). There thus appears to be distinct qualitative and quantitative differences in the pattern of adrenocortical secretion between young pony foals and the young of other species.

A distinct diurnal rhythm was already established at one day of age in foals in this investigation and became slightly more pronounced up to about one week of age. The mean afternoon plasma cortisol concentration during the first twenty-two days of life was 60.6% of that found in morning samples. The difference between morning and afternoon values was highly significant and equated very closely with the diurnal rhythm of adult ponies. Reports of similar studies in domestic animals are lacking, but Franks (80) noted that mature diurnal trends in man were not present until one-to-three years of age.

The rapid attainment of plasma cortisol concentrations equivalent to those of adult ponies and the rapid establishment of a mature diurnal rhythm suggests that the pituitary-adrenal function of foals is well-developed from an early age. This adaptation to extra-uterine life occurs much more rapidly than in neonatal man and may be a reflection of the obvious differences between these two species with regard to the maturity of a number of body functions at birth.

Plasma cortisol concentrations were significantly depressed and the normal diurnal rhythm eliminated by administration of dexamethasone (DXM) to foals between one and nine days of age. This effect became maximal on the fifth day of treatment and mean cortisol levels remained below .3 µg/dl from that time until at least ten hours after the last dexamethasone injection. Cortisol concentrations continued to be depressed, and in many cases were undetectable, on the seventh, eighth and ninth days of therapy despite the progressive reduction in DXM dosage from four, to three to two to one milligram during this period. This finding agrees well with the observation that even small doses of DXM can maximally suppress adrenocortical secretion in the horse (97). Plasma cortisol levels began to rise by twenty-four hours after the last

DXM injection, but were still lower than those of control animals at forty-eight hours. Concentrations continued to rise through each succeeding sampling interval before the diurnal rhythm was re-established three days after cessation of therapy (day 12).

Samples drawn on the third and eighth days after steroid withdrawal demonstrated a "rebound" in cortisol levels to significantly higher values than were found in control foals. In addition, the diurnal rhythm at these times was present but of altered character. Afternoon plasma cortisol concentrations in treated foals were 84% of the morning values as compared to 55% of morning values in non-treated foals. By the thirteenth day after cessation of DXM administration cortisol levels and diurnal trends in treated animals and controls were essentially identical.

Several workers have demonstrated that even a single dose of DXM is capable of depressing plasma cortisol concentrations for more than twenty-four hours in adult horses (97,105,173). Hoffsis et al. (97) noted that levels were already reduced by one hour after such treatment, suppression being maximal at twelve to twenty-four hours, and values did not return to normal for seventy-two hours. Acute dexamethasone treatment is also known to produce similar effects in cattle (73), sheep (18, 45), dogs (90,111,201) and man (113,125,145,211).

It is not known why DXM-treated foals in this study took five days to develop maximal adrenal suppression and similar studies in young animals of other species are not available for comparison. It seems unlikely that foals metabolize and excrete dexamethasone more rapidly than do adult horses. It is, however, possible that foals require higher circulating DXM levels for adrenal suppression than do adults and that successive dosages produce cumulative effects which become maximal after five days. This could also explain the fact that cortisol levels did not rise at the end of the treatment period despite reduction of the DXM dose. A further consideration is that DXM was administered at 8 am each day, that is, at about the time of the expected peak morning ACTH and cortisol surge. Studies in man have

demonstrated that the central mechanism controlling ACTH release requires higher glucocorticoid levels for suppression at this time than at other times of day (162).

The "rebound" of plasma cortisol concentrations to higher than normal levels, after the termination of a chronic course of exogenous GCS administration, has not previously been reported. It has been demonstrated that pituitary levels and release of ACTH are reduced during prolonged GCS therapy in man (155,178), and that the adrenal response to exogenous ACTH is also reduced in horses (96,173) and sheep (18). In addition, the hypothalamic formation and release of CRF may be retarded by GCS (173). It is also possible that the hypothalamic sensitivity to circulating GCS levels is reduced during steroid therapy and that, after abrupt withdrawal, circulating cortisol levels rise in response to pituitary ACTH release. If the threshold at which plasma cortisol concentrations negatively feed-back on CRF and ACTH release was higher than normal, then elevated cortisol levels would be tolerated. Plasma cortisol concentrations could only return to normal when normal hypothalamic and pituitary sensitivity were restored. Another possibility is that adrenal sensitivity to ACTH is increased immediately after steroid withdrawal, but it would be expected that the resulting increased plasma cortisol levels would negatively feed-back to reduce ACTH secretion if the hypothalamic and pituitary sensitivity to circulating cortisol concentrations was normal.

The rapid return of foal plasma cortisol concentrations to normal or supranormal levels after cessation of dexamethasone administration indicates that the pituitary-adrenal secretory mechanism did not suffer permanent damage. A similar conclusion has been drawn from the studies of asthmatic children whose adrenal function rapidly recovered after termination of a course of steroid therapy lasting a mean of seven years (155). Robinson et al. (178) showed that plasma cortisol levels returned to normal in most patients within forty-eight hours after the last of a series of GCS treatments lasting one-to-eighteen months. In contrast, the same workers and Graber et al. (88) showed that a

proportion of such patients may require an extended period for recovery of normal adrenocortical function and during this time may show signs of adrenal insufficiency. Extrapolation from these and similar studies has led to the belief that prolonged steroid therapy frequently leads to secondary adrenocortical insufficiency in performance horses (118,164), a condition often referred to as the "turning out syndrome" (197). This assumption has not been backed by quantitation of plasma cortisol concentrations and is not well-substantiated by the results of the current investigation. More prolonged studies in adult horses will be required before this hypothesis can be proven.

Foals given equine rhinopneumonitis virus at four days of age had slightly higher mean daily plasma cortisol concentrations during the period of the investigation than did control animals, irrespective of their dexamethasone treatment status. In addition, two foals that died after showing signs of severe respiratory distress following ERV infection, and one foal that died from an E. coli septicemia, showed a marked terminal rise in plasma cortisol levels prior to death and a loss of the diurnal rhythm. Values in the latter foal on the morning prior to death were about eight times higher than those of control foals on the same day. These results indicate that foals are capable of mounting a substantial glucocorticoid response to the stress of infection without experiencing terminal adrenocortical failure.

Nathanielsz et al. (160) and Rossdale et al. (182) noted a similar marked elevation in cortisol levels in full-term foals suffering lactic acidosis and postnatal convulsions shortly after birth. Analogous effects have also been observed in calves which developed an infectious diarrhea, both before and after signs appeared (101,133). In addition, the very high cortisol concentrations found in children with meningitis could not be further increased by exogenous ACTH administration indicating that the adrenals were already maximally stimulated in this disease state (180).

Very high plasma cortisol concentrations have been reported with acute medical and surgical illnesses in adult horses (96) and man

(104,121,129), the highest levels being found in the most acute conditions. Jacobs and Nabarro (104) considered this to be a natural response to stress and concluded that it may be the result of defective hepatic metabolism as well as increased adrenocortical secretion of cortisol in response to hypothalamic stimulation produced by neural inputs.

## B. Clinocopathologic Findings

The four pony foals inoculated intravenously with virulent Army strain #183 ERV developed a severe clinical disease syndrome after an incubation period of twenty-four to forty-eight hours. A period of pyrexia was characterized by maximum rectal temperatures of 103.2 to 104°F on the fifth postinoculation day. One-to-three days after the onset of pyrexia, clinical signs of upper respiratory tract disease became apparent and rapidly increased in severity. At this time, there was clear evidence of pneumonia and pleural effusion and the resultant extreme respiratory distress proved fatal to two (50%) of the animals on the fifth and seventh postinoculation days, respectively. Despite the severe involvement of the respiratory tract in this disease syndrome, coughing was not a major clinical feature.

The major pathological lesions in these foals were referable to involvement of the respiratory tract and lymphoid system. Pulmonary lesions included diffuse congestion and edema with focal areas of emphysema and edema. In addition, there was a marked effusion of fibrin-flecked fluid into the pleural cavity. Histiocytic cells in the submucosal lymphoid tissues of the small intestine of one foal contained characteristic eosinophilic inclusion bodies similar to those reported in the hepatic, alveolar, and bronchial cells of ERV-infected aborted fetuses (14,26,44) and in the bronchial epithelial cells of neonatal foals succumbing to ERV infection (57).

The incubation period, degree of pyrexia, and initial clinical signs observed in the four experimental foals inoculated with virulent ERV were very similar to those previously described by Doll et al. (59) after

inoculation of weanling horses with ERV. However, the later development of severe pneumonic signs and hydrothorax was not encountered by Doll et al., and other workers agree that pneumonia is a rare occurrence in older foals and adults infected with ERV (26). Similarly, the high mortality rate (50%) encountered in the current investigation is atypical (14,26), since pathological lesions are normally confined to the upper respiratory mucosa (14) and the resultant clinical disease is mild (14,26,59,170).

Pneumonia and death have, however, been reported in young foals following natural infection with ERV (26) and a related "early neonatal death syndrome" is thought to be due to the in utero infection of the fetus late in gestation (14,26,36,57,170). Affected foals were either weak at birth, or become so within forty-eight hours, and were soon unable to stand or nurse (57). The heart and respiratory rates were markedly elevated and harsh vesicular respiratory sounds and sibilant rales were heard on auscultation. Marked dyspnea and respiratory distress became more severe and death resulted a few hours to three days later (57). The pathologic lesions were remarkably similar to those observed in the two foals that died in the current investigation, but pleural effusion was not a common finding. In addition, eosinophilic intranuclear inclusions were found in the bronchiolar epithelium of some foals with mild to moderate necrotizing bronchiolar lesions (57).

Similar lesions are found in fetuses aborted as a result of ERV infection of the gestating mare (14,26,44,103,170,222) but in this case, excessive fluid accumulation in the body cavities, as occurred in the experimental foals, is a consistent pathological finding (14,26,44, 170). Edema of the lungs and other organs and transudation of fluid into the body cavities of aborted fetuses is thought to be due to the leakage of plasma protein resulting from widespread necrotizing vascular lesions (103). A similar mechanism was probably active in the four ERV-inoculated foals in this study since all showed a consistent reduction in plasma protein concentrations. This feature was most marked in one of the foals that died (2377); the plasma protein level

falling from 6.9 g % on the day of infection to 4.8 g % when death occurred five days later.

The widespread vascular lesions found in aborted fetuses (103) are probably responsible for the necrosis, degeneration and hemorrhage seen in many organs including the lymphoid tissues (14,44). Degeneration of the germinal centers of the spleen and lymph nodes, and generalized lymph node congestion, edema, and hemorrhage were also evident in the two foals which died as a result of ERV inoculation in this study. In addition, the thymus of one foal was grossly atrophied. Bryans et al. (36) described similar lesions, along with massive necrosis of all elements of the thymus, in foals which died after developing signs of severe respiratory distress, reddened mucosae, and diarrhea, with rapidly developing lethargy and weakness, during the first week of life. These foals exhibited clinical signs and postmortem lesions referable to secondary bacterial infection and, in addition, they developed a marked lymphopenia and neutrophilic leukocytosis during the course of the disease. ERV was isolated from about 1/3 of these foals which led to the suggestion that ERV infection of the fetus late in gestation, by invoking destruction of the major lymphocyte populations, increases the susceptibility of neonatal foals to a variety of infections. Bryans et al. (36) hypothesized that foals which escaped unresolvable thymic damage and were able to compensate immunologically for splenic damage would survive and develop normally. They further speculated that failure of full reconstitution of immunological capability could lead to an immunodeficiency syndrome which could eventually prove fatal. The evaluation of both the humoral and cell-mediated immune systems of the two foals that survived severe ERV infection in this study do not support this latter hypothesis but the fact remains that the two foals which died did display degenerative lymphoid lesions.

One of the ERV-inoculated foals developed a transient neurological disturbance thirteen to fifteen days postinoculation, after having apparently recovered from the pneumonic form of the disease. Clinical signs consisted of posterior incoordination with hypermetria, dysmetria

and generalized tremors. Excitement precipitated clonic convulsions, characterized by opistotonus and extensor rigidity, followed by recovery either within a few seconds or more than one minute later. Neither weakness nor pyrexia were prominent features in this foal.

A similar clinical syndrome has not been reported in young foals, but neurological disturbances have been observed in older animals in association with natural outbreaks of abortion and respiratory disease (40,131,187,200) and following experimental inoculation of both virulent (102,103) and vaccinal ERV (132). The predominant clinical signs encountered in these animals were posterior ataxia, progressing to total paresis in some cases, along with an inability to urinate and defecate (200). Other findings have included lethargy and edema of the hind limbs (40), stiffness and reluctance to move (102), urinary incontinence (131,132), sensory nerve deficits, and total paralysis, except for the head, without loss of pain sensation or anal reflexes (131). In some animals the signs have been transient, but in others progressive deterioration has necessitated euthanasia (200). Elevated protein levels have been consistently observed in the CSF (102,103,132) and are compatible with the overriding pathological lesion which reflects a nonsupportive disseminated meningoencephalomyelitis involving the brain and spinal cord (40,102,103,130,131), secondary to necrotizing vascular lesions (103).

The survival of the foal which developed a neurological disturbance in this investigation meant that tissues were not available for histopathological evaluation. The predominant clinical signs, however, likely reflect diffuse CNS lesions with definite involvement of the cerebellum. The lack of muscular weakness or paralysis indicate that the spinal cord was not significantly affected and thus this syndrome appears distinct from those reported above. It is also possible that it was not related to ERV inoculation.

None of the foals inoculated intravenously with three or five times the recommended dose of an attenuated live ERV vaccine[1] subsequently

[1] Rhinomune$^{R}$, Norden Laboratories Inc., Lincoln, Nebraska.

developed pyretic responses or any clinical disease signs. These results indicate that this vaccine is extremely safe and nonvirulent. Clinical trials conducted by other workers (14,26,170) support this view and, in addition, it has been demonstrated that the attenuated virus is not shed by vaccinated horses (14,26), thus avoiding the possibility of re-establishment of virulence. The vaccine did not induce a leukopenia in the foals evaluated here, nor in the animals studied by Bass et al. (14), and thus it is unlikely to predispose to bacterial infection.

The DXM-treated foals, which were not inoculated with either attenuated or virulent ERV, all developed mild to moderate clinical signs of an upper respiratory tract infection the day after cessation of DXM treatment. Coughing and a mucopurulent nasal discharge were the predominant features. In view of the fact that three of the four control foals, and none of the animals inoculated with attenuated ERV, developed similar signs, it appears that DXM treatment predisposed these animals to bacterial and/or viral respiratory tract infections. It seems likely that these infections did not become established until after several days of DXM treatment and thus did not reach clinical proportions until the end of the treatment period. It is now well-documented that prolonged GCS therapy can predispose man and animals to a variety of infections (51,151,206,213), increase the severity of existing infections (43,206), and cause recrudescence of latent and subclinical infections (53,192,206).

Correlation of the clinical signs with the results of laboratory evaluation of the humoral and cell-mediated immune systems of the DXM-treated foals will be discussed later.

One of the control foals used in this investigation died at five days of age as a result of an E. coli septicemia. Laboratory studies revealed that this foal had a plasma protein concentration of 5.1 g % at one day of age as compared to a mean value of 6.55 g % for all the other foals. Furthermore, this animal was the only one lacking detectable antibodies to either ERV or Brucella abortus at this time. It thus seems likely that failure of colostral antibody absorption predisposed this foal

to a fatal E. coli infection. Juelsgaard (112) also found that E. coli septicemia was a major problem in colostrum-deprived foals and these results serve to further emphasize the importance of passively-derived colostral antibodies in the prevention of bacterial diseases in neonatal foals.

## C. Hematologic Findings

The mean total leucocyte (wbc) counts of pony foals were low at one day of age (5911 $\pm$ 356/mm$^3$), but rose considerably during the first seventeen days of life such that values in twenty-two-day-old foals (9583 $\pm$ 833/mm$^3$) were significantly higher than those found in one day old animals. Similar studies involving young ponies are not available for comparison, but Sato et al. (186) reported on the leucogram changes in five thoroughbred (TB) foals sampled from birth to six months of age. Mean total wbc counts at birth (8,640 $\pm$ 2710/mm$^3$) were much higher than those recorded here but still increased slightly up to twenty days of age when values (9680 $\pm$ 3280/mm$^3$) were very similar to those of twenty-two day old foals in this study. The total wbc counts of TB and quarter horse (QH) foals investigated by Schalm et al. (190) were similarly high at birth (9600 $\pm$ 3372/mm$^3$) but changed very little during the first month of life. Todd et al. (214) found mean values of 8360 $\pm$ 519/mm$^3$ for TB foals one-to-four weeks of age.

The mean total neutrophil counts of one day old pony foals were low (3876 $\pm$ 307/mm$^3$) but increased rapidly during the first twelve to seventeen days of life so that values at twenty-two days of age (6612 $\pm$ 660/mm$^3$) were significantly higher than those found at one day of age. These findings differ markedly from those of Schalm et al. (190) who reported appreciably higher counts in TB and QH foals at birth (6962 $\pm$ 2760/mm$^3$) and also noted a slight overall decline in values over the first month of life. Sato et al. (186) also reported mean total neutrophil counts in TB foals at birth which were appreciably higher than those of the pony foals investigated here. An overall increase in counts over the first ten days of life followed a similar trend to

that of the pony foals but a marked variability between the individual animals made interpretation and extrapolation of results difficult.

The mean lymphocyte counts of one day old pony foals (1965 $\pm$ 138/mm$^3$) were again lower than those observed in newborn TB and QH foals (2192 $\pm$ 891/mm$^3$) by Schalm et al. (190) and in newborn TB foals (3223 $\pm$ 737/mm$^3$) by Sato et al. (186). In addition, lymphocyte counts tended to remain below those reported in TB and QH foals throughout the first three weeks after birth. A significant decline in lymphocyte counts occurred during the first four days of life in the pony foals investigated here, but this was followed by a marked increase so that the counts in twenty-two day old foals (2874 $\pm$ 313/mm$^3$) were significantly higher than those found at one and four days of age. An identical trend was found by Sato et al. (186) in TB foals and, while Schalm et al. (190) observed a similar general increase in counts with increasing age, their sampling times ranged enough to obscure short-lived alterations.

Pony foals in this study had neutrophil:lymphocyte ratios of approximately 2:1 at birth and the subsequently increased neutrophil counts coupled with reduced lymphocyte counts caused this ratio to widen to 3.6:1 at four days of age. Thereafter, neutrophil counts rose more slowly than lymphocyte counts and thus this ratio became narrower at seventeen days of age (2.7:1) and twenty-two days of age (2.3:1). Neutrophil:lymphocyte ratios of 1.6:1 and 2.8:1 have been reported in newborn TB (186) and TB and QH (190) foals, respectively, and are similar to that determined here for pony foals. However, "hot-blooded" foals show a rapid narrowing of this ratio with age as lymphocyte counts increase at a faster rate than neutrophil counts and thus a neutrophil:lymphocyte ratio of approximately 1:1, which is characteristic of young adult horses, is attained by one-to-two months of age (186, 190).

The above comparisons indicate that these are definite qualitative and quantitative differences between the major leukogram values of pony foals and foals of the "hot-blooded" breeds. Both the neutrophil and

lymphocyte counts of pony foals are lower at birth and, while neutrophil counts rise rapidly to approximate levels found in "hot-blooded" foals, lymphocyte counts rise more slowly and consequently neutrophil: lymphocyte ratios remain appreciably higher than those of "hot-blooded" foals at three weeks of age.

These differences are clinically important in the evaluation of blood samples obtained from young pony foals since the normal ranges quoted by laboratories and textbooks refer to adult rather than foal values and values derived from horses rather than ponies. A value could fall within the normal range for adult horses but be grossly abnormal in a pony foal, thus leading to dangerous clinical misinterpretations. Furthermore, ponies are frequently used in equine research with the results of these investigations being freely extrapolated to include "hot-blooded" horses on the assumption that both have identical physiological functions. This assumption is likely misguided and thus there is a great need, especially in the case of frequently used hematological data, to compile accurate ranges of normal values for ponies and to provide relevant comparisons with horse values.

The monocyte and eosinophil counts of pony foals investigated here were generally low and showed no significant trends with increasing age, results which are in good agreement with those reported by other workers in foals of TB and QH breeding (186,190,214).

The reported mean plasma fibrinogen concentration found in TB foals at birth is 265 mg% with an accepted range of 200-400 mg% (190). This agrees very well with the concentration found in the one day old pony foals studied here (mean 252 $\pm$ 32 mg%). Pony foals showed a rapid increase in plasma fibrinogen concentrations with age and consequently mean levels in twenty-two day old foals (475 $\pm$ 38 mg%) were significantly higher than mean levels in one day old foals. Schalm et al. (190) noted a similar trend in TB foals, mean values increasing to 365 mg% at one week of age and 430 mg% at one month of age. It has been suggested, however, that this trend likely does not represent a true age effect, but rather indicates a response to subclinical respiratory tract infections causing an inflammation-mediated rise in fibrinogen

concentrations (190). This may have been the case in the current study since a number of the foals developed mild signs of upper respiratory disease irrespective of whether they were inoculated with ERV or not.

Mean plasma protein concentrations were slightly higher in one day old pony foals than those reported in TB foals, but were within the accepted normal range (190). Levels declined somewhat during the first twelve days of life, but this was likely related to the frequent (twice daily) collection of blood during this period, since this trend was later reversed when sampling became less frequent. Additional evidence for this interpretation comes from the fact that TB foals show a general increase in plasma protein levels during the first month of life (190).

The administration of glucocorticosteroids to adult horses and ponies causes a relatively consistent pattern of leukogram changes characterized by leukocytosis with absolute neutrophilia, lymphopenia, and eosinopenia (2,6,140,167,205,206). These alterations have been noted within two hours after administration of a single intravenous dose of DXM (190,205) and the effects are generally maximal between seven and twenty-four hours posttreatment (6,190,205). Counts return to normal within forty-eight hours (6,205), or less (167,206), depending on the preparation used, the dose, and the route of administration. Neutrophil counts show a maximal increase of 96-185% (167) up to 300% (190, 205) and lymphocyte counts are depressed by 41 to 72% (167,190,205).

Eosinophil counts tend to fall more slowly than lymphocyte counts, the former showing maximal depression at about forty-eight hours with a return to normal counts ninety-six hours after DXM administration (6, 205).

The leukogram changes noted in man (76,78,228), dogs, cats, and cattle (190), and guinea pigs (11,77) following GCS administration are very similar to those noted in the horse except that monocytopenia is a consistent GCS-induced change in the former five species (78,168,190, 228) but not in the latter (140,167,190,205,206).

The reported leukogram changes induced by a chronic course of GCS administration are generally similar to those induced by a single

injection except that the changes persist throughout the treatment period (11,77). The maximal degree of lymphopenia is no greater than that observed following a single dose and the kinetics of the lymphopenia do not appear to be cumulative but rather are strictly related to each GCS dose (77).

Tarr and Olsen (206) reported that the classic GCS-induced leukogram changes persisted in adult horses throughout a four day course of DXM treatment with return to normal counts within thirty-six hours after the last injection. Magnuson et al. (140), noted, however, that while lymphopenia and eosinopenia persisted throughout a nine day course of daily DXM injections, neutrophil counts fell to below baseline values after a marked neutrophilia during the initial three days. Similarly, Archer (6) reported a return of leukocyte counts towards normal, after an initial marked neutrophilia and lymphopenia, despite continued dosing with hydrocortisone and ACTH at twelve hour intervals.

Pony foals in the current investigation were bled at one day of age, before any DXM treatments were given, and again on the fourth and seventh days of such therapy and after treatment had ceased. Total leukocyte, total neutrophil, and lymphocyte counts were not significantly altered by DXM administration although all three had slightly higher values in treated foals than in nontreated foals. These trends tended to be reversed after cessation of therapy. The band neutrophil, monocyte, and eosinophil counts were all low and no influence of DXM could be detected by the methods employed.

The results of this investigation contrast markedly with similar studies performed in adult horses and ponies, and in the other species described above. It is possible that foals are inherently more resistant to the effects of DXM on their circulating leukocytes than are adult horses, or that the effects of the drug did not last a full twenty-four hours. This latter possibility could be very important in that samples were collected at about 8 am each day, that is a full twenty-four hours after the previous DXM injection. However, this view is not supported by the results of plasma cortisol determinations;

cortisol concentrations remained markedly depressed during this period indicating that the effects of DXM did, indeed, last a full twenty-four hours. In addition, others have found leukogram changes to be still maximal twenty-four hours after administration of DXM in adult horses (190,205).

It is possible, as has already been noted in some studies in adult horses (6,140), that leucocyte counts were markedly altered during the first two or three days of DXM treatment and then later returned towards baseline values, in spite of continued therapy, so that samples collected on the fourth and seventh days failed to reveal any appreciable effects. Such a phenomenon could be related to the development of tolerance to high circulating GCS levels, induced by a yet unexplained mechanism.

A further possible explanation could lie in the fact that DXM depressed endogenous cortisol concentrations to very low levels. The dose of DXM used (4 mg) may have simply acted as replacement for deficient endogenous cortisol and not increased the total circulating GCS levels sufficiently to alter leucocyte kinetics. Again, this possibility is unlikely in that lower DXM doses, on a mg/kg basis, have induced profound leukogram alterations in older hoses (167,205, 206).

The increased circulating neutrophil counts induced by the administration of glucocorticosteroids has been shown to result from release of mature neutrophils from the marginated pool contained in small blood vessels (190,205) and bone marrow (24) as well as decreased migration of neutrophils into the tissues (24). Neonatal pony foals have relatively low neutrophil counts and possibly also a minimal marginated neutrophil pool from which to draw. If this was the case, they would not be capable of responding to steroid therapy with increased neutrophil counts.

A major finding in this investigation was the fact that total leukocyte, total neutrophil, and lymphocyte counts were all significantly lower at birth than at twenty-two days of age and most of this

increase occurred during the first week of life. It is very likely that the natural stimulus of increasing age was much stronger than the stimulus of DXM treatment and the former "masked" the effects of DXM. This effect may have been especially marked in the case of lymphocyte counts which were slightly higher in the treated group whereas a reduction in counts would have been expected.

It is not known which of the above mechanisms were active in the DXM-treated foals, but it is not unreasonable to suggest that a combination of these were responsible for the observed effects.

The only significant hematological alteration induced by DXM during the current investigation was a marked reduction in the plasma fibrinogen concentration of foals on both the fourth and seventh treatment days. This effect has not been previously investigated in foals and no similar results have been observed in adult horses (190). However, this result might be expected based on the fact that increased plasma fibrinogen levels are the result of inflammatory processes, especially those involving the respiratory epithelium (190) and an accepted action of glucocorticosteroids is suppression of inflammation (43,79,213,230).

The increase in plasma fibrinogen concentrations with increasing age noted in the control foals may, indeed, have been related to mild upper respiratory infections, the inflammatory effects of which were suppressed by DXM in treated foals, resulting in lower plasma fibrinogen concentrations in this latter group. The fact that fibrinogen levels rose rapidly after cessation of DXM therapy, and were indeed higher than those of control foals during this time, supports the proposed hypothesis. An alternative explanation lies in the fact that GCS exert a catabolic action on proteins (190) of which fibrinogen is one. However, no similar reduction in plasma protein levels was noted suggesting that this mechanism likely did not play a major role in the depression of plasma fibrinogen concentrations.

Equine rhinopneumonitis virus inoculation did not significantly alter

leucocyte counts in the pony foals studied, although the total white blood cell, total neutrophil, and lymphocyte counts were all somewhat lower in virus treated foals three days after inoculation than in control animals of equivalent age. Over the next fourteen days, values became slightly higher, for all three of these leucogram parameters, in the virus treated group than in the control group.

Doll et al. (59) demonstrated a leukopenia, persisting for up to thirteen days, in foals inoculated with virulent ERV. This was largely due to a marked neutropenia, other blood elements remaining relatively unaffected. Similar leukogram changes have been reported in ERV-infected foals affected by the early neonatal death syndrome (57). These effects were more marked than those noted in the present study, but Doll et al. (59) used older foals and again it is likely that the natural trends of leukogram alteration with increasing age obscured the effects of ERV in this investigation. In addition, Doll et al. (59) used an inoculum of virulent ERV whereas most of the pony foals studied here were instead given a large intravenous dose of attenuated live ERV and, it has been previously demonstrated that this attenuated virus does not induce leukopenia (14). All four foals which were inoculated with virulent ERV in this study developed severe clinical signs of infection and two died, but none of them demonstrated the characteristic leukogram alterations described by Doll et al. (59), adding further credence to the postulation that age changes produced an overriding effect.

The plasma fibrinogen concentrations of foals inoculated with either live or attenuated ERV were very similar to those of control animals and increased with age during the period of investigation. These results are somewhat surprising in view of the above postulated mechanism for elevation of plasma fibrinogen levels (190) and the marked respiratory signs induced by inoculation of virulent ERV. However, a number of control foals showed signs of mild upper respiratory tract disease, and many probably had subclinical infections, which may account for this apparent paradox.

Plasma protein concentrations were significantly higher in virus-

inoculated foals than in controls and this may have reflected increased gamma-globulin concentrations due to the antibody response to the virus. This is unlikely, however, in that humoral responses were generally poor, and, in addition, virus-treated foals had appreciably higher plasma protein levels than controls even before viral inoculation. This result most probably reflects normal experimental variation between individuals.

### D. Humoral Immune Function

Many of the pony foals investigated here had appreciable anti-ERV neutralizing antibody and anti-brucella agglutinating antibody titers at one day of age. Since these closely approximated the titers found in the respective dams, they were assumed to be of colostral origin. It has been shown previously that foals lack detectable specific circulating antibodies at birth, but after ingestion of antibody-rich colostrum, titers rise and reach levels very similar to those of their dams by one to two days of age (3,33,109,112). The intestinal mechanism of colostral gamma-globulin absorption is most highly active during the first few hours of life, after which time its activity rapidly declines and is negligible by 24 to 36 hours of age (34,107,108,112).

ERV neutralizing antibody titers declined progressively during the first five weeks of life in foals not inoculated with ERV, and this decline did not appear to be significantly influenced by DXM administration. Foals inoculated with ERV at four days of age showed a continued reduction in mean titers eight days later. However, titers subsequently rose and, although they did not reach preinoculation levels, they were significantly higher than those of noninoculated foals indicating that a primary humoral response to the virus had occurred. When all the foals were vaccinated with a modified live ERV product[1] at between 35 and 40 days of age, titers rose within ten days and remained elevated, at a similar level, seven days later. Foals not previously inoculated

[1] Rhinomune$^{R}$, Norden Laboratories Inc., Lincoln, Nebraska.

with ERV showed a less marked and less prolonged response to vaccination than foals in the ERV-inoculated group. These characteristics indicate that the foals inoculated with ERV at four days of age developed an anamnestic response following vaccination whereas the titer rise in control foals following vaccination was more characteristic of a primary humoral response.

The colostral anti-brucella agglutinin titers of foals varied widely but declined preogressively up to three days of age, at which time Brucella abortus bacterin was subcutaneously administered to all foals. Eight days later mean titers were elevated and this rise continued up to seventeen days of age, followed by a leveling off in later samples.

Twelve-day-old foals had anti-SRBC hemagglutinating titers of 1:2 or less at the time of intravenous inoculation of a SRBC suspension. All animals developed significantly increased titers ten days later followed by a decline to lower levels at thirty-five days of age.

The above results provide conclusive evidence that foals as young as three days of age are capable of responding to challenge with a variety of antigens by mounting significant humoral antibody responses and developing sensitization for subsequent anamnestic responses. These findings contrast markedly with the apparent immunological unresponsiveness or hyporesponsiveness of newborn and very young animals of several other species (198,202). However, support for the results of this investigation comes from the work of Juelsgaard (112) who reported that both colostrum-fed and colostrum-deprived foals immunized with tetanus toxoid, killed Brucella abortus, bovine serum albumen, and killed eastern and western equine encephalomyelitis viruses at one or two weeks of age, developed marked humoral responses to these antigens. In addition, B-cell sensitization was evidenced by a more rapid and sustained titer rise following booster vaccination four-to-eleven weeks later.

In the same study, it was noted that there were some age-related effects on antibody production. Foals vaccinated for the first time at four or eight weeks old tended to show slightly more rapid titer rises

and slightly higher peak titers to certain antigens than foals vaccinated at an earlier age. It seems likely that a number of factors could contribute to this maturation of humoral immune responses with increasing age. Some workers believe that there is an increase in the "antigen-sensitive" B-lymphocyte population with increasing age (141,212). This may be related to repeated exposure to homologous or heterologous cross-reacting antigens and thus be an acquired adaptive mechanism (112). It is also possible that the ability to process antigens for the development of antibody responses may increase during early neonatal life. This important function is carried out by macrophages and T-lymphocytes and is especially important in the development of humoral responses to thymus-dependent antigens such as soluble proteins (20).

The Ig M class of antibodies is the first to increase following antigen stimulation in young foals (135,183) and in many other species (172). The ability to produce Ig G appears later and more gradually and could further explain the slightly increased antibody producing capabilities with aging (112).

Pre-existing antibodies of colostral origin appeared to interfere somewhat with the humoral responses of foals in this study although this effect could not be accurately quantitated due to the small number of animals available. This effect was, however, quite variable; some foals with anti-ERV neutralizing antibody titers of 1:4 or less showed no titer increase following ERV inoculation, whereas one animal with a titer of 1:16 developed an eight-fold increase. Less variability was apparent in the case of colostral antibody-induced inhibition of responses to Brucella abortus vaccination. Titers ranged from 1:5 to 1:1280 at one day of age and all foals with titers of less than 1:80, when vaccinated with killed Brucella abortus bacteria at three days of age, subsequently developed increased specific antibody levels. Only one foal with a pre-vaccination titer of 1:160 or more developed a positive serological response.

In spite of the variation between individual animals it is obvious that the presence of colostral antibodies inhibited the development of

primary humoral responses in foals, the highest titers causing the most marked inhibition. This situation has also been observed in calves (94, 203), lambs (12,17), pigs (100), and foals (3,112,188,189).

Alexander and Mason (3) noted that foals did not develop humoral responses to horsesickness vaccine and were protected from infection with virulent virus during, and sometimes after, the period when specific colostrum-derived antibodies were present in their circulation. Similarly, Schützler (188,189) reported that foals born of dams hyperimmunized with tetanus toxoid showed no response when immunized at less than three weeks of age. However, a second inoculation of tetanus toxoid given a few weeks later resulted in a rapid rise in antitoxin titers indicating that sensitization had occurred following the primary vaccinal dose.

Juelsgaard (112) demonstrated that the humoral responses of foals, given primary immunizations with a variety of antigens at between one and eight weeks of age, were slower developing and weaker the higher the level of maternal antibody present at the time of vaccination. While the peak response of seronegative ten-week-old foals to killed Brucella abortus occurred at one week postvaccination, the presence of maternal antibody delayed this peak until the third postimmunization week. Foals with high maternal tetanus antitoxin titers showed no response to primary immunization with tetanus toxoid although some sensitization had occurred as indicated by increased titers within one week of a later booster dose. Similarly, the higher the antibody titer, the less marked was the anamnestic response to booster vaccination. This may have been a direct effect of antibody on the secondary responses similar to that exerted on the primary response and/or it could also represent a restriction of the initial sensitization induced by the primary dose. A similar effect was also noted with responses to human serum albumin and killed WEE and EEE viruses.

The above work (112) and that of others (154,184,188,189,218) indicates that passive antibody is more effective in inhibiting the primary antibody response than the sensitization of the humoral immune system for

the development of an anamnestic response, although such inhibition has been demonstrated (152,153,217). It is probable that less antigen is required for sensitization and the elicitation of secondary responses than is needed for the development of primary antibody formation. Thus, while high levels of passive antibody could bind with and remove the requisite amount of antigen and prevent development of a primary response, a small quantity of antigen could remain to stimulate sensitization of immunocompetent B-lymphocytes. These proliferate to produce the so-called specific "memory cells" which can be "recalled" on subsequent exposure to the homologous antigen, thereby rapidly initiating an anamnestic humoral response.

The implications of the above findings with regard to the routine immunization of foals will be discussed later.

The inhibitory effects of pre-existing antibodies are not limited to those of passively derived colostral antibodies, since actively produced immunoglobulins exert a similar effect. In the case of ERV, Neely and Hawkins (161) observed diminished responses to vaccination in horses of all ages with neutralizing titers of more than 1:64 at the time of vaccination. Similarly, Bryans (35) demonstrated that animals with preinfection titers of greater than 1:100 failed to develop anamnestic antibody responses, viremia, or clinical signs of infection when challenged.

The nature of the immune responses to ERV, especially the age-related effects, is not clearly understood, as indicated previously (literature review). In general, young horses and foals mount poor humoral responses, irrespective of the pre-existing antibody titer (66,85,161). The findings of this investigation support this view in that none of the foals developed a greater than eight-fold titer increase following ERV inoculation or subsequent booster vaccination and many did not develop an antibody titer rise in spite of low preinoculation titers. It is interesting to note that the two foals which showed the greatest titer increases were those given virulent rather than vaccinal virus.

Dutta and Shipley (66) noted that one-to-four month old foals developed negligible responses to either primary or booster doses of a modified live ERV vaccine regardless of their exact age or pre-existing anti-ERV antibody titers. An analogous pattern was observed by Neely and Hawkins (161) in two-to-seven month old foals subjected either to vaccination or natural challenge. In contrast, mares in the same study developed very rapid, one-to-five-fold, titer increases indicative of an anamnestic response. Furthermore, Gerber et al. (85) were unable to demonstrate appreciable serological responses to a two dose ERV vaccination series given to six-to-eight month old foals, whereas pregnant mares and eighteen-to-twenty-one month old horses developed four-to-sixty-four-fold titer increases.

The poor humoral responses of foals to inoculation with vaccinal ERV more likely reflect a lack of previous exposure to the virus than an immaturity of the immune system of the young horse (85,161). The apparent low antigenicity of the virus may necessitate multiple exposures before appreciable sensitization and good humoral responses are attained. An additional consideration is the widespread distribution of ERV (14,26,44,66,161) which means that a large proportion of the horse population have demonstrable titers (9,59) and consequently most foals possess anti-ERV antibodies of colostral origin. It is possible that relatively low titers will inhibit the serological responses of foals to ERV, whereas higher titers are needed to elicit this effect in older horses. This hypothesis could only be substantiated by studies using sero-negative animals which are, to say the least, difficult to find.

The decline in titers of anti-brucella, anti-ERV and anti-SRBC antibodies of colostral or endogenous origin, was not significantly influenced by DXM administration in the foals studied. These findings are in agreement with those of Butler and Rossen (37) who showed that the plasma survival, plasma clearance and urinary excretion of at least IgG were not altered by even high dose GCS therapy in man.

The serological responses of foals to ERV, killed Brucella abortus, and SRBC were not altered either during or after DXM therapy. Similarly,

many studies in man have failed to demonstrate GCS-induced suppression of antibody formation when therapeutic dosage rates were employed (230). However, administration of GCS in high dosages for three-to-five days has been shown to significantly reduce serum concentrations of IgG and sometimes other Ig classes in human samples drawn thirty and, in some cases, up to ninety days later (37). In addition, it has been demonstrated that high in vitro steroid concentrations will reduce antibody production by isolated lymph node fragments from rabbits immunized against diptheria toxoid and BSA (4). The earliest phases of antibody production appear to be more vulnerable than the later stages (30,63,230) and the primary response seems to be more influenced than the anamnestic response (30,230). Thus, it has been concluded that these effects are mediated via inhibition of peripheral nonactivated B-lymphocytes (150) and possibly also "helper" T-cells (63) and since they are believed to be related to destruction of lymphocytes and inhibition of protein synthesis, marked effects would not be expected in steroid-resistant species such as the horse.

The lack of demonstrable DXM-induced alterations in antibody production by the foals investigated here does not rule out this possibility in that animal numbers were small and, in the case of ERV and Brucella abortus titers, colostral antibodies interfered somewhat with the interpretation of results.

### E. Cell-mediated Immune Function

The in vitro blastogenic responses of lymphocytes to antigens and certain plant mitogens are frequently used as monitors of cell-mediated immune (CMI) functions in the horse and other species (43,65,85,140, 151,205,209,226). Furthermore, the blastogenic transformation of equine lymphocytes in response to ERV antigen has been reported, indicating that CMI mechanisms may play a role in resistance to ERV infection (65,85, 209,226). The proposed effects of glucocorticosteroids on the CMI system remain controversial (79) and relatively few quantitative studies

have been performed in the horse (140,205,206).

Consequently, it was decided to investigate the ability of young foals to mount significant CMI responses and to evaluate the effects of ERV inoculation and chronic DXM treatment on these responses. Lymphocytes, harvested from foals at four, twelve, and seventeen days of age, were cultured in vitro with PHA, PWM, Con A, Anti-globulin (AG) and ERV.

It is generally believed that PHA specifically stimulates T-lymphocytes (43,50,193), while PWM stimulates both T- and B-cells (50, 193) and Con A induces proliferation of column-adherent T-cells (27). B-lymphocytes, by virtue of their surface immunoglobulin receptors, are the predominant cells stimulated by antiglobulin (50), while most of the current evidence suggests that antigen-induced blastogenesis is a property of T-cells (50).

Lymphocytes, collected from foals as young as four days of age, showed appreciable blastogenesis in response to PHA, Con A, and PWM, while the response to AG was somewhat poorer. Responses to the three plant mitogens showed wide interindividual variation but mean values were of a similar magnitude to those of older foals and adult mares evaluated concurrently (Kaeberle, M. L., 1977, personal communication, Department of Veterinary Microbiology and Preventative Medicine, Iowa State University, Ames, Iowa). Similary, the mitogen stimulation results agree well with values previously reported for older ponies and horses (65,140,205,206) and two-week-old gnotobiotic foals (209). The antiglobulin responses of four-day-old foals were generally poor (mean S.I. less than 2.0) but were improved by the time foals were twelve and seventeen days of age, and at that time were similar to the mean values for adult pony mares studied concurrently (Kaeberle, M.L., 1977, personal communication, Department of Veterinary Microbiology and Preventative Medicine, Iowa State University, Ames, Iowa.

It thus appears, based on the above in vitro studies, that the cell-mediated immune functions of foals are fully active from an early age.

Using similar techniques, Thomson and Mumford reached an identical conclusion (209).

The AG responses at four days of age may indicate a lack of maturity of B-cell function at this time, but this conclusion can not be drawn on the basis of the above results, since the number of animals studied was small and many of them were receiving concurrent DXM treatment which, in itself, produced significant effects.

Lymphocytes collected from foals before ERV inoculation and those from noninoculated animals demonstrated negligible in vitro responses to ERV (mean S.I. less than 2.0). Eight and thirteen days postinoculation, however, lymphocyte responses were increased in terms of all three stimulation criteria used. When values from these two sampling days were pooled, both the net counts per minute and $\log_{10}$ S.I. were significantly higher in ERV-inoculated foals than in controls. These results provide very good evidence that young foals are capable of responding to ERV infection by mounting a CMI response in addition to the humoral response noted previously.

Thomson and Mumford (209) reported results which were remarkably similar to those noted in this investigation, in terms of the magnitude of the in vitro responses of lymphocytes from conventional foals to ERV. No blastogenic response occurred before the foals were inoculated with low passage ERV at four-to-six months of age. Sensitization developed within one-to-two weeks and peaked two-to-three weeks postinfection. The stimulation indices were between 1.6 and 3.6 at that time but declined to lower levels over the next three to five weeks. Two week old gnotobiotic foals, however, showed a more prolonged period of sensitization with peak responses many times greater than those of conventional foals. This latter result likely reflects more the gnotobiotic state of these foals rather than their age and these workers speculated that these foals either exhibited delayed responses or experienced prolonged exposure to the virus. The increased magnitude and duration of the responses seems to support the latter hypothesis.

In an investigation attempting to determine the effects of age and

pregnancy on the relative magnitudes of the CMI and humoral responses of horses, Gerber et al. (85) observed significantly higher stimulation indices in ERV-vaccinated, six-to-eight-month-old foals, than in non-vaccinated controls. Good responses were already present one week postvaccination but fell to low levels by five weeks. A more pronounced and prolonged response was seen following a booster vaccination, the peak occurring at eight weeks. However, the humoral responses of these foals were poor as compared to those of eighteen-to-twenty-one-month old foals and adults. These latter two groups, on the other hand, had poor CMI responses. In general, it appeared that the humoral and CMI responses were inversely correlated and animals did not develop good CMI and good humoral responses at the same time. Others have observed a similar effect (209,226) and this may be due to the blockage of antigenic determinants on T-cells by high levels of antibody and may serve as a regulatory mechanism (85).

As noted previously, young horses, in general, do not develop good humoral responses following infection or vaccination with ERV (66, 85,161). It is unlikely that this indicates an immaturity of the humoral immune system because good antibody responses have been noted in very young foals to a variety of antigens in this and other investigations (112). More likely this reflects the relatively poor antigenicity of the virus, a lack of prior exposure to it and probably also the blocking effect of colostral antibodies in young foals. This study has conclusively demonstrated that foals as young as four days of age do develop both humoral and cell-mediated immune responses to vaccinal as well as virulent ERV and that the attenuated live virus vaccine,[1] even when administered intravenously at very high doses, does not induce clinical illness or leukogram changes in very young foals or affect their ability to respond to other antigens. It would seem that much could be gained, in terms of protection aginst ERV infection, from vaccinating foals at an early age, even immediately after birth, rather than waiting until they are two-to-four-months-old as is

[1]Rhinomune$^{R}$, Norden Laboratories Inc., Lincoln, Nebraska.

customary. Furthermore, the protection afforded by vaccination should not be gauged in terms of humoral responses alone since this only tells part of the story.

When the stimulation data were expressed in terms of net cpm it was found that, eight days post-ERV inoculation, foals had significantly poorer blastogenic responses to PHA, Con A, and PWM than did control foals of the same age. These trends were not evident when the results were presented as stimulation indexes or $\log_{10}$ S.I. and are thus probably of doubtful significance. However, it has been reported (209) that live ERV inhibits the uptake of $^{3}$H-thymidine by unsensitized foal lymphocytes cultured with PHA and it is well-known that following infection ERV replicates in, and is carried by, circulating mononuclear cells of the intact animal (35). It is possible that eight days post-inoculation the virus was still present in circulating lymphocytes and that this may have inhibited the metabolic processes of the lymphocytes when cultured in vitro and reduced their uptake of $^{3}$H-thymidine.

The blastogenic responses of foal lymphocytes to Con A, PWM, and AG were significantly lower in DXM-treated foals than in control animals on the fourth day of such therapy, when the results were expressed in terms of net cpm. The S.I. and $\log_{10}$ S.I. of AG responses were also reduced at this time and remained so eight days after the last DXM injection. Since AG stimulation is considered to be a measure of B-cell activity (50), and PWM induces blastogenesis in both T- and B-cells (50,193), it would appear that B-lymphocyte function was affected to a greater extent than T-lymphocyte function. The lack of effects on PHA-stimulation, coupled with a reduction in Con A stimulation may indicate that only a certain subpopulation of T-cells were influenced by DXM treatment.

These results agree well with those of Targowski (205) who observed that PHA-induced blastogenesis of lymphocytes from adult ponies was unaffected by DXM administered in doses slightly lower than those employed in this study. He concluded that PHA-responsive lymphocytes are relatively unaffected by steroids. Magnuson et al. (140) demonstrated that

high (1 mg/kg) doses of DXM caused a marked reduction in the number of circulating lymphocytes in adult ponies but did not affect the in vitro responses of the remaining lymphocytes to PHA. These workers provided evidence that most of these remaining lymphocytes bore surface receptors for immunoglobulin and $C_3$ indicating that there was a greater reduction in circulating T-cell numbers than B-cell numbers. In the current investigation, however, circulating lymphocyte numbers were not reduced by DXM treatment and no attempt was made to differentially quantitate the T- and B-cell populations.

Reports concerning the effects of DXM on antiglobulin-induced lymphocyte blastogenesis in man and animals are not available but, as noted above, it is generally agreed that circulating B-cell numbers are proportionally less reduced than T-cell numbers by steroid treatment (76,77,78,140,168,228). If this was the case in the foals studied in this investigation then it would appear that DXM either interfered with the B-cell antiglobulin receptors or with metabolic processes within these cells. An alternative explanation may lie in the fact that lymphocyte counts in the foals were low at one day of age and increased gradually to adult levels during the first three weeks of life. Since T-cells are thought to make up about 80% of the circulating lymphocyte pool, even a marked reduction in B-cell numbers would not greatly alter the already low total lymphocyte counts but would appreciably reduce the in vitro B-cell-mediated antiglobulin responses. Differential quantitation of B- and T-lymphocytes would be necessary before this latter hypothesis could be proven.

Tarr and Olsen (206) demonstrated that during a four day course of daily therapeutic doses of DXM, the in vitro responses of adult horse lymphocytes to Con A and PWM were reduced by 43% and 29%, respectively, results which agree well with those derived for foals in this study. These same workers also showed, however, that PHA responses were reduced by 48% during the same time. This latter finding is in conflict with those made here and with those of the other investigations cited above (104,205). Similarly, studies in man (76) have shown that PHA-induced

blastogenesis is relatively unaffected by in vitro doses of GCS which markedly depress Con A and PWM responses, thus indicating selective depletion of certain lymphocyte subpopulations (78,79). On the other hand, GCS treatment does not appear to influence the in vitro responses of guinea pig lymphocytes to PHA, Con A, or PWM in spite of a marked lymphopenia, which suggests that all lymphocyte subpopulations are equally depressed (11,77). Further confusion is introduced by the report by Muscoplat et al. (158) who found that DXM caused a marked temporary reduction in the response of calf lymphocytes to PHA with a return to normal responses before cessation of the course of therapy.

In addition to the obvious species differences in the in vitro lymphocyte blastogenic responses noted above, it seems likely that the variation in culture methods employed by different investigators is responsible for at least some of the confusing discrepancies which have been reported (79). Fauci et al. (79) concluded that, while glucocorticosteroids do suppress CMI responses, direct suppression of lymphocyte proliferation is unlikely to be the major mechanism involved. Rather, decreased access of lymphocytes to the sites of antigenic deposition, decreased effects of soluble mediators on other cell types, and many of the other recognized effects of GCS on numerous processes may be of greater importance than their controversial effects on lymphocyte blastogenesis.

It is tempting to speculate that the increased incidence of signs of upper respiratory tract disease seen in the DXM-treated foals was due to depression of CMI rather than humoral responses in these foals. While humoral antibody responses of the young foals were not measurably affected by DXM, their in vitro lymphocyte blastogenic responses to antiglobulin were. This may indicate that B-cell responses were indeed suppressed and that the in vitro studies may have been a more reliable guide to this than the measurement of antibody titers which would not detect subtle alterations in B-cell function. In addition, local secretory antibody production may be of prime importance in the prevention of respiratory disease, and if this process were to be inhibited

by steroid administration, increased susceptibility to infections could result. Nasal secretory antibody titers were not measured in this investigation and such determinations would be necessary to test this hypothesis.

Furthermore, viral-induced synthesis and release of interferon has been shown to be reduced by steroid administration in some species (81,116). Interferon is thought to play a significant role in the local protection against viral infection (13). The role of interferon, or the effects of GCS thereon, have not been investigated in horses to determine if these effects are, indeed, of any significance.

# VI. SUMMARY

A two-part study was conducted to investigate the effects of dexamethasone (DXM) and equine rhinopneumonitis virus (ERV), alone or in combination, on the adrenocortical function and immunological responsiveness of young pony foals. The ability of young pony foals to mount humoral and cell-mediated immune responses to antigenic challenge was also studied and baseline values established. Similarly, the pattern of development of mature adrenocortical function in young foals was evaluated by comparison of the cortisol levels of foals with adult pony and horse values.

A radioimmunoassay kit procedure, designed for use on human plasma, was used to measure cortisol concentrations in the plasma of adult horses and ponies and young pony foals. The results of four validation experiments indicated that this kit was well-suited for use on equine plasma after slight modification of the assay protocol.

The mean daily plasma cortisol concentration observed in adult ponies (2.74 µg/dl) was similar to that of adult horses (3.00 µg/dl) and equivalent to that previously reported (124). Cortisol levels varied considerably from day-to-day and between individuals, but an overall pattern of diurnal variation was very evident with early morning levels being much higher than late afternoon levels. The adrenocortical function of foals was apparently very mature at birth since concentrations at one day of age were only slightly lower than adult levels and showed only a small increase during the first three weeks of life. In addition, foals exhibited a well-developed diurnal variation in cortisol levels at one day of age and this continued throughout the investigation.

Dexamethasone (DXM) rapidly induced a profound depression in the plasma cortisol concentrations, and obscured the normal diurnal rhythm, when administered by daily intramuscular injection to foals during the first nine days of life. This suppression persisted throughout the period of DXM treatment despite gradual reduction in the dosage used. Levels

began to rise within twenty-four hours of the last dose and subsequently "rebounded" temporarily to supranormal levels, during which time the normal diurnal rhythm was gradually re-established. There was no evidence that chronic DXM treatment induced persistent adrenocortical insufficiency.

Foals inoculated with ERV at four days of age developed slightly higher cortisol levels than noninoculated foals. In addition, three foals which became clinically ill and died during the investigation showed a very marked terminal rise in cortisol concentrations indicating that the pituitary-adrenal axis of young foals is capable of mounting a very marked response to the stress of infection, without terminal failure.

The mean total leukocyte, neutrophil, and lymphocyte counts at one day of age were very much lower than the accepted values for these parameters in adult horses (190). Apart from an initial decline in lymphocyte counts during the first four days of life, all three parameters showed a significant increase in values with increasing age, before "leveling off" to adult values at seventeen-to-twenty-two days of age. There was a progressive diminution in the neutrophil: lymphocyte ratio after the fourth day of life as lymphocyte counts increased more slowly, but to a proportionally greater extent, than neutrophil counts. Plasma fibrinogen concentrations also increased significantly as foals aged; from a mean of 252 mg% at one day of age to a mean of 475 mg% at twenty-two days of age. This latter increase was attributed to the establishment of a flora of microorganisms in the respiratory tracts of these foals and the concommitant inflammation resulting from mild or subclinical infections.

The in vitro blastogenic responses of foal lymphocytes to the plant mitogens, PHA, Con A, and PWM, were equivalent, at four days of age, to the responses observed in older foals and adults. This indicates that the proliferative capabilities of T-lymphocytes are well-developed in ponies at a very early age. In addition, young foals developed rapid T-cell sensitization to ERV antigen, since in vitro blastogenic responses

to ERV were increased by eight days post-ERV inoculation. The responses of lymphocytes to *in vitro* stimulation with AG, a predominantly B-cell stimulator, suggests that the humoral immune system was less well-developed in four-day-old foals than in older foals. However, the good serological responses of three-day-old foals to vaccination with killed *Brucella abortus* strain 19 indicates that foals do, indeed, possess relatively mature antibody-forming capabilities at less than one week of age.

The presence of colostral antibodies interfered somewhat with the serological responses of foals to both *Brucella abortus* and ERV antigens. This inhibition increased as the preexisting titer increased, but many foals developed a postinoculation titer rise. In addition, a sensitization of the humoral immune system following ERV inoculation was evidenced by a greater and more sustained (anamnestic) response following later booster vaccination. A further indication of the humoral immune competence of young foals comes from the finding that twelve-day-old foals showed eight-to-sixteen-fold titer rises following intravenous immunization with SRBC at twelve days of age.

No adverse clinical reactions or leukogram alterations were induced by intravenous inoculation of four-day-old foals with three-to-five times the recommended dose of an attenuated ERV vaccine,[1] indicating that this product is very safe and reaction free. In addition, the sensitization of both the humoral and cell-mediated immune systems following such inoculation demonstrates that very young foals are capable of responding immunologically to ERV vaccination and thus nothing is to be gained by delaying primary immunization, unless titers of maternal origin are very high.

In contrast to the lack of clinical signs induced by inoculation of foals with attenuated ERV, all four foals inoculated intravenously with virulent ERV developed a severe clinical syndrome, characterized by fever, depression, rhinitis, pneumonia and fibrinous pleural effusion, after an incubation period of twenty-four to forty-eight hours. Two

[1] Rhinomune$^{R}$, Norden Laboratories Inc., Lincoln, Nebraska.

of the foals died after a period of severe respiratory distress, and of the two that survived, one developed a transient neurological syndrome indicative of cerebellar dysfunction. Lymphoid lesions consisting of degenerative changes in the lymph node germinal centers were evident in the two foals which died.

Most of the foals which were treated with DXM developed mild upper respiratory tract infections, characterized by mucopurulent nasal exudates and coughing, within one or two days after cessation of DXM therapy. This suggests that suppression of the immune system of the foals was induced by DXM, allowing infections to become established. The *in vitro* response of B-lymphocytes to AG was suppressed during, and for some time after, the period of DXM therapy, which indicates that this mechanism may have been responsible for the increased susceptibility to infections. The humoral antibody responses to antigens were not, however, depressed in DXM-treated foals, but these may not have been as subtle a guide to humoral capabilities as the *in vitro* responses of B-cells. Furthermore, inhibition of local antibody production in the respiratory tract may be of significance in this context, but this aspect of immune function was not investigated here.

Dexamethasone therapy caused only equivocable and temporary inhibition of some of the *in vitro* responses of lymphocytes to plant mitogens and thus it appears that inhibition of CMI functions is not a major action of glucocorticosteroids in young foals.

DXM did not induce any significant alterations in the major circulating leukocyte populations but did markedly depress plasma fibrinogen concentrations which probably reflects its action in inhibiting inflammation.

In conclusion, this study failed to demonstrate any persistent suppressive effects of either equine rhinopneumonitis virus or dexamethasone, or a combination thereof, on the cell-mediated or humoral immune system of pony foals, although degenerative lymphoid lesions were evident in the two foals fatally infected with virulent ERV.

# VII. BIBLIOGRAPHY

1. Abraham, G. E. 1974. Radioimmunoassay of steroids in biological materials. Acta Endocrinol. Suppl. 183:4-42.

2. Alexander, F., and R. W. Ash. 1955. The effect of emotion and hormones on the concentration of glucose and eosinophils in horse blood. J. Physiol. (Lond.) 130:703-710.

3. Alexander, R. A., and J. H. Mason. 1941. Studies on the neurotropic virus of horsesickness. VII. Transmitted immunity. Onderstepoort J. Vet. Sci. Anim. Ind. 16:19-32.

4. Ambrose, C. T. 1964. The requirement for hydrocortisone in antibody-forming tissues cultivated in serum-free medium. J. Exp. Med. 119:1027-1049.

5. Andersen, V., F. Bro-Rasmussen, and K. Hougaard. 1969. Autoradiographic studies of eosinophil kinetics; effect of cortisol. Cell Tissue Kinet. 2:139-146.

6. Archer, R. K. 1957. The mechanism of eosinopenia produced by ACTH and corticoids in the horse. J. Pathol. Bacteriol. 74: 387-395.

7. Atkinson, J. P., and M. M. Frank. 1973. Effect of cortisone therapy on serum complement components. J. Immunol. 111:1061-1066.

8. Atkinson, J. P., and A. D. Schreiber, and M. M. Frank. 1973. Effect of corticosteroids and splenectomy on the immune clearance and destruction of erythrocytes. J. Clin. Invest. 52:1509-1517.

9. Bagust, T. J., R. R. Pascoe, and T. J. Harden. 1972. Studies on equine herpesviruses. Aust. Vet. J. 48:47-53.

10. Balow, J. E., and A. S. Rosenthal. 1973. Glucocorticoid suppression of macrophage migrating inhibitory factor. J. Exp. Med. 137:1031-1041.

11. Balow, J. E., D. L. Hurley, and A. S. Fauci. 1975. Immunosuppressive effects of glucocorticosteroids: Differential effects of acute vs. chronic administration on cell mediated immunity. J. Immunol. 114:1072-1076.

12. Barr, M., A. T. Glenny, and J. W. Howie. 1953. Active immunization of ewes and their lambs. J. Pathol. Bacteriol. 65:155-168.

13. Baron, S. and H. B. Levy. 1966. Interferon. Annu. Rev. Microbiol. 20:291-318.

14. Bass, E. P., R. L. Sharpee, and W. H. Beckenhauer. 1973. Equine viral rhinopneumonitis: Pathography and prophylaxis. Norden News 48:5-9.

15. Bassett, J. N., and N. T. Hinks. 1969. Micro-determination of corticosteroids in ovine peripheral plasma: Effect of venipuncture, corticotrophin, insulin and glucose. J. Endocrinol. 44:387-403.

16. Bassett, J. M., and G. D. Thorburn. 1969. Foetal plasma corticosteroids and the initiation of parturition in the sheep. J. Endocrinol. 44:285-286.

17. Batty, I., A. Thomson, and J. R. Hepple. 1954. The active immunization of newborn lambs against pulpy kidney disease (Clostridium welchii type D enterotoxemia). Vet. Rec. 66: 249-254.

18. Beaven, D. W., E. A. Espiner, and D. S. Hart. 1964. The suppression of cortisol secretion by steroids, and response to corticotrophin in sheep with adrenal transplants. J. Physiol. (Lond.) 171:216-230.

19. Becker, M. J., D. Helland, and D. N. Becker. 1976. Serum cortisol (hydrocortisone) values in normal dogs as determined by radioimmunoassay. Am. J. Vet. Res. 37:1101-1102.

20. Benecerraff, B., and P. G. H. Gell. 1959. Studies on hypersensitivity. I. Delayed and Arthus-type skin reactivity to protein conjugates in guinea pigs. Immunology 2:53-63.

21. Bennett, W. A. 1954. Histopathological alterations of adrenal and anterior pituitary glands in patients treated with cortisone. J. Bone. Jt. Surg. Am. 36-A:867-874.

22. Berenbaum, M. C., P. A. Fluck, and N. P. Hurst. 1973. Depression of lymphocyte responses after surgical trauma. Br. J. Exp. Pathol. 54:597-607.

23. Biesel, W. R., and M. I. Rapoport. 1969. Inter-relations between adrenocortical functions and infectious illness. N. Eng. J. Med. 280:541-546, and 596-604.

24. Bishop, C. R., J. W. Athens, and D. R. Boggs. 1968. Leukokinetic studies: XIII. A non-steady state kinetic evaluation of the mechanism of cortisone-induced granulocytosis. J. Clin. Invest. 47:249-260.

25. Bliss, E. D., A. A. Sandberg, H. Nelson, and K. Eik-Ness. 1953. The normal levels of 17-hydroxycorticosteroids in the peripheral blood of man. J. Clin. Invest. 32:818-823.

26. Blood, D. C., J. A. Henderson, and O. M. Radostits. 1979. Veterinary Medicine. 5th ed. Lea and Febiger, Philadelphia. 1135 pp.

27. Boldt, D., A. M. Skinner, and S. Kornfield. 1972. Studies of two subpopulations of human lymphocytes differing in responsiveness to concanavalin A. J. Clin. Invest. 51:3225-3229.

28. Bottoms, G. D., O. F. Roesel, F. D. Raush, and E. L. Akins. 1972. Circadian variation in plasma cortisol and corticosterone in pigs and mares. Am. J. Vet. Res. 33:785-790.

29. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. Suppl. 21:77.

30. Bradley, J., and C. J. Elson. 1971. Suppression of the immune response. J. Med. Genet. 8:321-340.

31. Breznock, E. M., and R. D. McQueen. 1970. Adrenal function during aging in the dog. Am. J. Vet. Res. 31:1269-1273.

32. Brock, P., E. W. Eldred, J. E. Woiszwillo, M. Doran, and H. J. Shoemaker. 1978. Direct solid-phase $^{125}I$ radioimmunoassay of serum cortisol. Clin. Chem. 24:1595-1598.

33. Bruner, D. W., P. R. Edwards, and E. R. Doll. 1948. Passive immunity in the newborn foal. Cornell. Vet. 38:363-366.

34. Bruner, D. W., E. R. Doll, F. E. Hull, and A. S. Kinkaid. 1950. Further studies on hemolytic icterus in foals. Am. J. Vet. Res. 11:22-25.

35. Bryans, J. T. 1969. On immunity to disease caused by equine herpesvirus I. J. Am. Vet. Med. Assoc. 155:294-300.

36. Bryans, J. T., T. W. Swerczek, R. W. Darlington, and M. W. Crowe. 1977. Neonatal foal disease associated with perinatal infection by equine herpesvirus I. J. Equine Med. Surg. 1:20-26.

37. Butler, W. T., and R. D. Rossen. 1973. Effect of methylprednisolone on immunoglobulin metabolism in man. Fed. Proc. 32:1028.

38. Campbell, J. R., and C. Watts. 1973. Assessment of adrenal function in dogs. Br. Vet. J. 129:134-145.

39. Campuzano, H. C., J. E. Wilkerson, P. B. Raven, T. Schabrm, and S. M. Horvath. 1973. A radioimmunoassay for cortisol in human plasma. Biochem. Med. 7:350-362.

40. Charlton, K. M., D. Mitchell, A. Girard, and A. H. Corner. 1976. Meningoencephalomyelitis in horses associated with equine herpesvirus I infection. Vet. Pathol. 13:59-68.

41. Chen, C. L., M. S. A. Kumar, M. D. Williard, and T. F. Liao. 1978. Serum hydrocortisone (cortisol) values in normal and adrenopathic dogs as determined by radioimmunoassay. Am. J. Vet. Res. 39:179-181.

42. Chretein, J. H., and V. F. Garagusi. 1971. Suppressed reduction of nitroblue tetrazolium by polymorphonuclear neutrophils from patients receiving steroids. Experimentia (Basel) 27:1343.

43. Claman, H. N. 1972. Corticosteroids and lymphoid cells. N. Eng. J. Med. 287:388-397.

44. Clark, T. L., and R. C. Dillman. 1974. Clinicopathology of equine rhinopneumonitis abortion in central Iowa. Vet. Med. Small Anim. Clin. 69:320-324.

45. Clements, A. S., and H. H. Newsome. 1973. Assay of cortisol, corticosterone, 11-deoxycortisol and cortisone in ovine peripheral and adrenal venous plasma. J. Endocrinol. 56: 413-419.

46. Cohen, I. R., L. Stavy, and M. Feldman. 1970. Glucocorticoids and cellular immunity in vitro: Facilitation of the sensitization phase and inhibition of the effector phase of a lymphocyte anti-fibroblast reaction. J. Exp. Med. 132:1055-1070.

47. Comline, R. S., L. W. Hall, R. B. Lavell, R. W. Nathanielsz, and M. Silver. 1974. Parturition in the cow: Endocrine changes in animals with chronically implanted catheters in the foetal and neonatal circulation. J. Endocrinol. 63:451-472.

48. Coons, A. H., E. H. Leduc, and J. M. Connolly. 1955. Studies on antibody production. I. A method for histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. J. Exp. Med. 102:49-59.

49. Crepea, S. B., G. E. Magnin, and C. V. Seastone. 1951. Effect of ACTH and cortisone on phagocytosis. Proc. Exp. Biol. Med. 77: 704-706.

50. Daguillard, F. 1972. Immunologic significance of in vitro lymphocyte responses. Med. Clin. N. Am. 56:293-303.

51. Dale, D. C., and R. G. Petersdorf. 1973. Corticosteroids and infectious diseases. Med. Clin. N. Am. 57:1277-1287.

52. Dalle, M., and P. Delost. 1976. Plasma and adrenal cortisol concentrations in foetal, newborn and mother guinea pigs during the perinatal period. J. Endocrinol.70:207-214.

53. Davies, D. H., and L. E. Carmichael. 1973. Role of cell mediated immunity in the recovery of cattle from primary and recurrent infections with infectious bovine rhinotracheitis virus. Infect. Immun. 8:510-518.

54. De Duve, C., R. Wattiaux, and M. Wibo. 1962. Effects of fat-soluble compounds on lysosomes in vitro. Biochem. Pharmacol. 9:97-116.

55. Dilliard, G. M., and P. Bodel. 1970. Studies on steroid fever. II. Pyrogenic and anti-pyrogenic activity in vitro of some endogenous steroids of man. J. Clin. Invest. 49:2418-2426.

56. Dimock, W. W., and P. R. Edwards. 1933. Is there a filterable virus of abortion in mares? Kentucky Agric. Exper. Sta. Bull. (Suppl.) 333.

57. Dixon, R. J., W. J. Hartley, D. R. Hutchins, E. E. Lepherd, C. Feilen, R. F. Jones, D. N. Love, M. Sabine, and A. L. Wells. 1978. Perinatal foal mortality associated with a herpesvirus. Aust. Vet. J. 54:103-105.

58. Dobson, H., and L. N. Kanchev. 1977. Effect of adrenal stimulation on bovine plasma steroids. J. Reprod. Fertil. 50:357-358.

59. Doll, E. R., M. E. Wallace, and M. G. Richards. 1954. Thermal, hematological and serological responses of weanling horses following inoculation of equine abortion virus: Its similarity to equine influenza. Cornell. Vet. 44:181-190.

60. Doll, E. R., and J. T. Bryans. 1963. Epizootiology of equine viral rhinopneumonitis. J. Am. Vet. Med. Assoc. 142:31-37.

61. Donohue, J., and D. Sgoutas. 1975. Improved radioimmunoassay of plasma cortisol. Clin. Chem. 21:770-773.

62. Drost, M., L. F. Kumagai, and M. Guzman. 1973. Sequential foetal-maternal plasma cortisol levels in sheep. J. Endocrinol.56:483-492.

63. Dukor, P., and F. M. Dietrich. 1968. Characteristic features of immunosuppression by steroids and cytotoxic drugs. Int. Arch. Allergy Appl. Immunol. 34:32-48.

64. Dunn, J. D., and A. J. Carrillo. 1978. Circadian variation in the sensitivity of the pituitary-adrenal system to dexamethasone suppression. J. Endocrinol. 76:63-66.

65. Dutta, S. K., and D. L. Campbell. 1977. Cell mediated immunity in equine herpesvirus type I infection. 1. In vitro lymphocyte blastogenesis and serum neutralization antibody in normal parturient and aborting mares. Can. J. Comp. Med. 41:404-408.

66. Dutta, S. K., and W. D. Shipley. 1975. Immunity and the level of neutralization antibodies in foals and mares vaccinated with a modified live-virus rhinopneumanitis vaccine. Am. J. Vet. Res. 36:445-448.

67. Dvořák, M. 1972. Adrenocortical function in foetal, neonatal and young pigs. J. Endocrinol. 54:473-481.

68. Eberhart, R. J., and J. A. Patt. 1971. Plasma cortisol concentrations in newborn calves. Am. J. Vet. Res. 32:1921-1927.

69. Ebert, R. H., and W. R. Barclay. 1952. Changes in connective tissue reaction induced by cortisone. Ann. Intern. Med. 37: 506-518.

70. Eisenstein, A. B. 1967. The adrenal cortex. 1st ed. Little, Brown and Company, Boston, Mass. 685 pp.

71. Ellis, G., and R. Morris. 1978. A one-tube micromethod for radio-immunoassay of plasma cortisol. Clin. Chem. 24:1954-1957.

72. Elissdale, M. H., H. R. Crookshank, and I. L. Berry. 1976. Diurnal variation in cortisol and other blood factors. J. Anim. Sci. 43:282.

73. Evans, L. E. 1973. Physio-pathologic studies in induced parturition in the cow (Bos taurus). Ph.D. Thesis. Iowa State University. 217 pp. University Microfilms, Ann Arbor, Mich. (Diss. Abstr. Int. 34:5250-B).

74. Fahmy, D., G. F. Read, and S. G. Hillier. 1975. Some observations on the determination of cortisol in human plasma by radio-immunoassay using antisera against cortisol-3-BSA. Steroids 26:267-281.

75. Farmer, R. W., and C. E. Pierce. 1974. Plasma cortisol determination: Radioimmunoassay and competitive protein binding compared. Clin. Chem. 20:411-414.

76. Fauci, A. S., and D. C. Dale. 1974. The effect of in vivo hydrocortisone on subpopulations of human lymphocytes. J. Clin. Invest. 53:240-246.

77. Fauci, A. S. 1975. Mechanism of corticosteroid action on lymphocyte subpopulations. I. Redistribution of circulating T and B lymphocytes to the bone marrow. Immunology 28:669-680.

78. Fauci, A. S. 1976. Mechanism of corticosteroid action on lymphocyte subpopulations. II. Diffferential effects of in vivo hydrocortisone, prednisone and dexamethasone on in vitro expression of lymphocyte function. Clin. Exp. Immunol. 24:54-62.

79. Fauci, A. S., D. C. Dale, and J. E. Balow. 1976. Glucocorticosteroid therapy: Mechanisms of action and clinical considerations. Ann. Intern. Med. 84:304-315.

80. Franks, R. C. 1967. Diurnal variation of plasma 17-hydroxycorticosteroids in children. J. Clin. Endocrinol. Metab. 27: 75-78.

81. Fulton, R. W., and B. D. Rosenquist. 1976. In vitro interferon production by bovine tissues: Effects of hydrocortisone. Am. J. Vet. Res. 37:1493-1495.

82. Gabal, A. A. 1977. Corticosteroids-side effects and toxicity. Proc. Amer. Assoc. Equine Pract. 23:393-396.

83. Gabrielsen, A. E., and R. A. Good. 1967. Chemical suppression of adaptive immunity. Adv. Immunol. 6:91-229.

84. Ganong, W. F., L. C. Alpert, and T. C. Lee. 1974. ACTH and the regulation of adrenocortical secretion. N. Engl. J. Med. 290: 1006-1011.

85. Gerber, J. D., A. E. Marron, E. P. Bass, and W. H. Beckenhauer. 1977. Effect of age and pregnancy on the antibody and cell mediated immune responses of horses to equine herpes virus I. Can. J. Comp. Med. 41:471-478.

86. Gerwurz, H., P. R. Wernick, P. G. Quie, and R. A. Good. 1965. Effects of hydrocortisone succinate on the complement system. Nature (Lond.) 208:755-757.

87. Gleason, M. H., and C. Ropke. 1969. Quantitative studies on cortisol-induced decay of lymphoid cells in the thymolymphatic system. Acta Pathol. Microbiol. Scand. 76:376-382.

88. Graber, A. L., R. L. Ney, W. E. Nicholson, D. P. Island, and G. W. Little. 1965. The natural history of pituitary-adrenal recovery following long-term suppression with corticosteroids. J. Clin. Endocrinol. Metab. 25:11-16.

89. Hafs, H. D., G. D. Niswender, P. V. Malven, C. C. Kaltenbach, R. G. Zimbelman, and R. J. Condon. 1977. Guidelines for hormone radioimmunoassays. J. Anim. Sci. 45:927-928.

90. Halliwell, R. E. W., R. M. Schwartzman, L. Hopkins, and D. McEvoy. 1971. The value of plasma corticosteroid assays in the diagnosis of bushings disease in the dog. J. Small Anim. Pract. 12: 453-462.

91. Hammond, J. E., J. C. Phillips, B. E. Statland, and P. Winkel. 1976. Physiological day-to-day variation of serum thyroxine and cortisol. Clin. Chem. 22:1171.

92. Hansen, H. J., and S. J. Holst. 1950. Fall av virusabort ohs sto i sverige. Nord. Veterinaermed. 2:131-159.

93. Hartley, L. H., J. W. Mason, R. P. Hogan, L. G. Jones, T. A. Kotchen, E. H. Mougey, F. E. Wherry, L. L. Pennington, and P. T. Rickets. 1972. Multiple hormonal responses to graded exercise in relation to physical training. J. Appl. Physiol. 33:602-606.

94. Henning, M. W. 1953. Calf parathyroid III. The transmission of antibodies to newly-born calves. Onderstepoort J. Vet. Res. 26:45-59.

95. Hillman, D. A., and G. J. P. Giroud. 1965. Plasma cortisone and cortisol levels at birth and during the neonatal period. J. Clin. Endocrinol. Metab. 25:243-248.

96. Hoffsis, G. F., and P. W. Murdick. 1970. The plasma concentrations of corticosteroids in normal and diseased horses. J. Am. Vet. Med. Assoc. 157:1590-1594.

97. Hoffsis, G. F., P. W. Murdick, V. L. Tharp, and K. Ault. 1970. Plasma concentrations of cortisol and corticosterone in the normal horse. Am. J. Vet. Res. 31:1379-1387.

98. Holley, D. C., D. A. Beckman, and J. W. Evans. 1975. Effect of confinement on the circadian rhythm of ovine cortisol. J. Endocrinol. 65:147-148.

99. Holub, D. A., J. W. Jailer, J. I. Kitay, and A. G. Frantz. 1959. Direct and indirect estimation of pituitary adrenocorticotrophin reserves in man following adrenal steroid therapy. J. Clin. Endocrinol. Metab. 19:1540-155.

100. Horlein, A. B. 1957. The influence of colostrum on antibody response in baby pigs. J. Immunol. 78:112-117.

101. Hudson, S., M. Mullford, W. G. Whittlestone, and E. Payne. 1976. Plasma corticoid levels in healthy and diarrhoeic calves from birth to twenty days of age. Br. Vet. J. 132:551-556.

102. Jackson, T. A., and J. W. Kendrick. 1971. Paralysis of horses associated with equine herpesvirus I infection. J. Am. Vet. Med. Assoc. 158:1351-1357.

103. Jackson, T. A., B. I. Osburn, D. R. Cordy, and J. W. Kendrick. 1977. Equine herpesvirus I infection of horses: Studies on the experimentally induced neurologic disease. Am. J. Vet. Res. 38:709-719.

104. Jacobs, H. S., and J. D. N. Nabarro. 1969. Plasma 11-hydroxycorticosteroid and growth hormone levels in acute medical illnesses. Br. Med. J. No. 5657:595-598.

105. James, V. H. T., M. W. Horner, M. S. Moss, and A. E. Rippon. 1970. Adrenocortical function in the horse. J. Endocrinol. 48:319-335.

106. Jeffcoate, S. L. 1977. Recent developments in steroid radioimmunoassays. J. Reprod. Fertil. 51:267-272.

107. Jeffcott, L. B. 1971. Duration of permeability of the intestine to macromolecules in the newly-born foal. Vet. Rec. 88:340-341.

108. Jeffcott, L. B. 1973. The mechanism of transfer of maternal immunity to the foal. Pages 419-435 in J. T. Bryans and H. Gerber, eds. Proc. third conf. equine infectious diseases, Paris 1972. S. Karger. Basel.

109. Jeffcott, L. B. 1974. Some practical aspects of the transfer of passive immunity to newborn foals. Equine Vet. J. 6:109-115.

110. Jennings, J. F., and G. Taylor. 1964. Effects of hydrocortisone hemisuccinate on immune lysis of sheep erythrocytes. Nature (Lond.) 203:661.

111. Johnston, S. D., and E. C. Mather. 1978. Canine plasma cortisol (hydrocortisone) measured by radioimmunoassay: Clinical absence of diurnal variation and results of ACTH stimulation and dexamethasone suppression tests. Am. J. Vet. Res. 39: 1766-1770.

112. Juelsgaard, S. G. 1975. Humoral antibody response in the neonatal foal. M.S. Thesis. Iowa State University. 190 pp.

113. Kao, M., S. Voina, A. Nichols, and R. Horton. 1975. Parallel radioimmunoassay for plasma cortisol and 11-deoxycortisol. Clin. Chem. 21:1644-1647.

114. Kass, E. H., and M. Finland. 1958. Corticosteroids and infections. Adv. Intern. Med. 9:45-80.

115. Kehlet, H., and C. Binder. 1973. Alterations in distribution volume and biological half-life of cortisol during major surgery. J. Clin. Endocrinol. Metab. 36:330-333.

116. Kilbourne, E. D., K. M. Smart, and B. A. Pokorny. 1961. Inhibition by cortisone of the synthesis and action of interferon. Nature (Lond.) 190:650-651.

117. Killiam, D. B., H. A. Garverick, and B. N. Day. 1973. Peripheral plasma progesterone and corticoid levels at parturition in the sow. J. Anim. Sci. 37:1371-1375.

118. Kirk, M. D. 1974. Field diagnosis and treatment of secondary adrenocortical insufficiency in the horse. Vet. Med. Small Anim. Clin. 69:1383-1386.

119. Kirkpatrick, J. F., L. Wiesner, C. B. Baker, and M. Angle. 1977. Diurnal variation of the plasma corticosteroids in the wild horse stallion. Comp. Biochem. Physiol. 57A:179-181.

120. Klein, G. P., M. Baden, and C. J. P. Giroud. 1973. Quantitative measurement and significance of five plasma corticosteroids during the perinatal period. J. Clin. Endocrinol. Metab. 36: 944-950.

121. Korpassy, A., H. Stoeckel, and P. Vecsei. 1972. Investigation of hydrocortisone secretion and aldosterone excretion in patients with severe prolonged stress. Acta Anaesthesiol. Scand. 16:161.

122. Kress, F. 1941. Virusabortus bei der Stute. Wein. Tierärztl. Monatsschr. 28:320.

123. Kress, F. 1944. Ueber virusbedingten Abortus bei Pferdestuten in den Donagauen. Ztschr. f. Infektionshrank Parasitäre Krank. u. Hyg. der Haustiere 60:180-193.

124. Kumar, M. S. A., T. F. Liao, and C. L. Chen. 1976. Diurnal variation in serum cortisol in ponies. J. Anim. Sci. 42:1360.

125. Kumar, M. S., A. M. Safa, and S. D. Deodhar. 1976. Radioimmunoassay for serum cortisol with $^{125}$I-labelled ligand: Comparison of three methods. Clin. Chem. 22:1845-1849.

126. de Lacerda, L., A. Kowarski, and C. J. Migeon. 1973. Integrated concentration and diurnal variation of plasma cortisol. J. Clin. Endocrinol. Metab. 36:227-238.

127. de Lacerda, L., A. Kowarski, and C. J. Migeon. 1973. Diurnal variation of the metabolic clearance rate of cortisol. Effect on measurement of cortisol production rate. J. Clin. Endocrinol. Metab. 36:1043-1049.

128. Laurie, M. B. 1960. The reticuloendothelial system, cortisone and thyroid function; their relation to native resistance to infection. Ann. N.Y. Acad. Sci. 88:83-98.

129. Lewell, M. J., S. R. Stitch, J. R. W. Dykes, and J. J. L. Ablett. 1970. Adrenal cortical function in patients with tetanus. Brit. J. Anaesth. 42:531-534.

130. Liptrap, R. M., and J. I. Raeside. 1978. A relationship between plasma concentrations of testosterone and corticosteroids during sexual and aggressive behavior in the boar. J. Endocrinol. 76:75-85.

131. Little, P. B., and J. Thorsen. 1976. Disseminated necrotizing myeloencephalitis: A herpes-associated neurological disease of horses. Vet. Pathol. 13:161-171.

132. Liu, K. M., and W. Castleman. 1977. Equine posterior paresis associated with equine herpesvirus I vaccine in California: A preliminary report. J. Equine Med. Surg. 1:397-401.

133. Lopez, G. A., R. W. Phillips, and L. D. Lewis. 1975. Plasma corticoid changes during diarrhea in neonatal calves. Am. J. Vet. Res. 36:1245-1247.

134. Macadaman, W. R., and R. J. Eberhart. 1972. Diurnal variation in plasma corticosteroid concentration in dairy cattle. J. Dairy Sci. 55:1792-1795.

135. McGuire, T. C., and T. B. Crawford. 1973. Passive immunity in the foal: Measurement of immunoglobulin classes and specific antibody. Am. J. Vet. Res. 34:1299-1303.

136. McGuire, T. C., K. L. Banks, D. R. Evans, and M. J. Poppie. 1976. Agammaglobulinemia in a horse with evidence of functional T-lymphocytes. Am. J. Vet. Res. 37:41-46.

137. McNatty, K. P., M. Cashmore, and A. Young. 1972. Diurnal variation in plasma cortisol levels in sheep. J. Endocrinol. 54:361-362.

138. McNatty, K. P., and A. Young. 1973. Diurnal changes of plasma cortisol levels in sheep adapting to a new environment. J. Endocrinol. 56:329-330.

139. Magnuson, N. S., K. L. Banks, and T. C. McGuire. 1976. The effect of corticosteroids on equine blood lymphocytes. Fed. Proc. 35:589.

140. Magnuson, N. S., T. C. McGuire, K. L. Banks, L. E. Perryman. 1978. In vitro and in vivo effects of corticosteroids on the peripheral blood lymphocytes from ponies. Am. J. Vet. Res. 39: 393-398.

141. Makinodan, T., and W. J. Peterson. 1964. Growth and senescence of the primary antibody-forming potential of the spleen. J. Immunol. 93:886-896.

142. Mandell, G. L., W. Rubin, and E. W. Hook. 1972. The effect of an NADH oxidase inhibitor (hydrocortisone) on polymorphonuclear leukocyte bactericidal activity. J. Clin. Invest. 51:615-624.

143. Manninger, R., and J. Csontos. 1941. Equine virus abortus der Stuten. Deut. Tierärtzl. Wchnschr. 49:105.

144. Mattingly, D. 1962. A simple fluorimetric method for the estimation of free 11-hydroxycorticoids in human plasma. J. Clin. Pathol. (Lond.) 15:374-379.

145. Melby, J. C. 1971. Assessment of adrenocortical function. New Eng. J. Med. 285:735-739.

146. Mendelson, J., and M. Finland. 1966. Effects of cortisol on interferon production by mouse peritoneal leucocytes and spleen in vitro and in vivo. Proc. Soc. Exp. Biol. Med. 123:98-104.

147. Migeon, C. J., A. B. French, L. T. Samuels, J. Z. Bowers. 1955. Plasma 17-hydroxycortisol levels and leucocyte values in the rhesus monkey including normal variations and the effect of ACTH. Am. J. Physiol. 182:462-468.

148. Milkovic, K., and S. Milkovic. 1963. Functioning of the pituitary-adrenal axis in rats at and after birth. Endocrinology 73: 535-539.

149. Miller, D. R., and H. G. Kaplan. 1970. Decreased nitroblue tetrazolium dye reduction in the phagocytes of patients receiving prednisone. Pediatrics 45:861-865.

150. Miller, J. J., and L. J. Cole. 1967. Resistance of long-lived lymphocytes and plasma cells in rat lymph nodes to treatment with prednisone, cyclophosphamide, 6-mercaptopurine, and actinomycin D. J. Exp. Med. 126:109-125.

151. Mirsha, S. K., R. S. Sandhu, H. S. Randhawa, V. N. Damodaran, and S. Abraham. 1973. Effect of cortisone administration on experimental nocardiosis. Infect. Immun. 7:123-129.

152. Möller, G. 1963. Studies on the mechanisms of immunological enhancement of tumor homografts. III. Interaction between humoral isoantibodies and immune lymphoid cells. J. Nat. Cancer Inst. 30:1205-1226.

153. Möller, G. 1966. Biologicl properties of 19S and 7S mouse isoantibodies directed against isoantigens of the H-2 system. J. Immunol. 96:430-439.

154. Morris, A., and G. Möller. 1968. Regulation of cellular antibody synthesis: Effect of adoptively transferred antibody-producing spleen cells on cellular antibody synthesis. J. Immunol. 101:439-445.

155. Morris, H. G., and J. R. Jorgensen. 1971. Recovery of endogenous pituitary-adrenal function in corticosteroid treated children. J. Pediatr. 79:480-488.

156. Mulnix, J. A., and G. D. Niswender. 1977. Radioimmunoassay for serum cortisol in normal dogs. Pages 94-95 in Proc. 6th World Congr. of the World Small Animal Vet. Assoc., Royal Netherlands Vet. Assoc., Amsterdam.

157. Murphy, B. E. P. 1967. Some studies of the protein-binding of steroids and their application to the routine micro and ultra-micro measurement of various steroids in body fluids by competitive protein-binding radioassay. J. Clin. Endocrinol. Metab. 27:973-990.

158. Muscoplat, C. C., R. E. Shope, A. W. Chen, and D. W. Johnson. 1975. Effects of corticosteroids on responses of bovine peripheral blood lymphocytes cultured with phytohemagglutinin. Am. J. Vet. Res. 36:1243-1244.

159. Nathanielsz, P. W., R. S. Comline, M. Silver, and R. B. Paisley. 1972. Cortisol metabolism in the fetal and neonatal sheep. J. Reprod. Fertil. Suppl. 16:39-59.

160. Nathanielsz, P. W., P. D. Rossdale, M. Silver, and R. S. Comline. 1975. Studies of fetal, neonatal and maternal cortisol metabolism in the mare. J. Reprod. Fertil. Suppl. 23:625-630.

161. Neely, D. P., and D. L. Hawkins. 1978. A two-year study of the clinical and serologic response of horses to a modified live-virus equine rhinopneumonitis vaccine. J. Equine Med. Surg. 2:532-540.

162. Nichols, T., C. A. Nugent, and F. H. Tyler. 1965. Diurnal variation in suppression of adrenal function by glucocorticoids. J. Clin. Endocrinol. Metab. 25:343-349.

163. Nowell, P. C. 1961. Inhibition of human leucocyte mitosis by prednisolone in vitro. Cancer Res. 21:1518-1521.

164. O'Connor, J. T. 1968. The untoward effects of corticosteroids in equine practice. J. Am. Vet. Med. Assoc. 153:1614-1617.

165. Oppenheim, J. J., B. G. Leventhal, and E. M. Hersh. 1968. The transformation of column-purified lymphocytes with non-specific and specific antigenic stimuli. J. Immunol. 101:262-270.

166. Orth, D. N., D. P. Island, and G. W. Liddle. 1967. Experimental alteration of the circadian rhythm in plasma cortisol (17-OHCS) concentration in man. J. Clin. Endocrinol. Metab. 27:549-555.

167. Osbaldiston, G. W., and J. H. Johnson. 1972. Effect of ACTH and selected glucocorticoids on circulating blood cells in horses. J. Am. Vet. Med. Assoc. 161:53-56.

168. Parrillo, J. E., and A. S. Fauci. 1978. Mechanisms of corticosteroid action on lymphocyte subpopulations. III. Differential effects of dexamethasone administration on subpopulations of effector cells mediating cellular cytotoxicity in man. Clin. Exp. Immunol. 31:116-125.

169. Pick, E., J. Brostoff, J. Krejci, and J. L. Turk. 1970. Interaction between "sensitized lymphocytes" and antigen in vitro. II. Mitogen-induced release of skin reactive and macrophage migration inhibitory factor. Cell. Immunol. 1:92-109.

170. Piersen, R. H. 1974. Clinical observations on planned infection and preventive vaccination for control of equine rhinopneumonitis. Proc. Amer. Assoc. Equine Pract. 19:69-74.

171. Porter, C. C., and R. H. Silber. 1950. A quantitative color reaction for cortisone and related 17, 21-dihydro-20-keto-steroids. J. Biol. Chem. 185:201-207.

172. Prokešová, L., J. Rejnek, J. Šterzl, and J. Trávníček. 1970. Ontogenic development of immunoglobulins in pigs. Pages 757-764 in J. Šterzl and I. Řiha, eds. Developmental aspects of antibody formation and structure. Academia, Publishing House of the Czechslovak Academy of Sciences, Prague.

173. Ray, R. S. 1977. ACTH and the corticosteroids. Proc. Amer. Assoc. Equine Pract. 23:407-410.

174. Reynolds, J. W. 1973. Serum total corticoid and cortisol levels in premature infants with respiratory distress syndrome. Pediatrics 51:884-890.

175. Reynoso, G. Plasma cortisol and urinary free cortisol. Pages 231-238 in N. R. Rose and H. Friedman, eds. Manual of clinical immunology. American Society for Microbiology. Washington, D.C.

176. Riegle, G. D., and J. E. Nellor. 1967. Changes in adrenocortical function during aging in cattle. J. Gerontol. 22:83-86.

177. Rinehart, J. J., S. P. Balcerzak, A. L. Sagone, and A. F. LoBuglio. 1974. Effects of corticosteroids on monocyte function. J. Clin. Invest. 54:1337-1343.

178. Robinson, B. N. B., S. Mattingly, and C. L. Cope. 1962. Adrenal function after prolonged corticosteroid therapy. Br. Med. J. No. 5292:1579-1583.

179. Rosenau, W., and H. D. Moon. 1962. The inhibitory effect of hydrocortisone on lysis of homologous cells by lymphocytes in vitro. J. Immunol. 89:422-426.

180. Rosenbloom, A. L., J. Savory, and J. H. Londono. 1971. The half-hour synthetic 1-24-corticotrophin test of adrenocortical reserve in children. J. Pediatr. 79:489-493.

181. Rosenquist, B. D. 1973. Induction and biological significance of interferon, with particular reference to cattle. J. Am. Vet. Med. Assoc. 163:821-824.

182. Rossdale, P. D., L. W. Hall, and P. W. Nathanielsz. 1973. Plasma cortisol in the foal during the late fetal and early neonatal period. Res. Vet. Sci. 15:395-397.

183. Rouse, B. T. 1971. The immunoglobulins of adult equine and foal sera: A quantitative study. Br. Vet. J. 127:45-51.

184. Rowley, D. A., and F. W. Fitch. 1964. Homeostasis of antibody formation in the adult rat. J. Exp. Med. 120:987-1005.

185. Saba, N. 1964. The estimation of cortisol and cortisone in bovine and ovine plasma. J. Endocrinol. 28:139-148.

186. Sato, T., K. Oda, and M. Kubo. 1978. Hematological and biochemical values of thoroughbred foals in the first six months of life. Cornell. Vet. 69:3-19.

187. Saxegaard, F. 1966. Isolation and identification of equine rhinopneumonitis virus (equine abortion virus) from cases of abortion and paralysis. Nord. Veterinaermed. 18:504-512.

188. Schützler, H. 1973. Untersuchungen zur Immunisierung von Fohlen gegen Tetanustoxin. II. Mitteilung: Übertragung von tetanus Antikörpern mit Kilostralmilch auf das neugeborene Fohlen und anschließende aktive Immunisierung. Arch. Exper. Vet. Med. 27:245-250.

189. Schützler, H. 1973. Untersuchungen zur Immunisierung von Fohlen gegen Tetanustoxin. III. Mitteilung: Aktive Immunisierung neugeborener und junger Fohlen von spezifisch vorbehandeten Muttertieren. Arch. Exper. Vet. Med. 27:251-262.

190. Schalm, O. W., N. C. Jain, and E. J. Carroll. 1975. Veterinary hematology. 3rd ed. Lea and Febiger, Philadelphia. 807 pp.

191. Shaw, K. E., and R. E. Nichols. 1962. The influence of age upon circulating 17-hydroxycorticosteroids of cattle subjected to blood sampling and exogenous adrenocorticotrophic hormone and hydrocortisone. Am. J. Vet. Res. 23:1217-1218.

192. Sheffy, B. E., and S. Rodman. 1973. Activation of latent infectious bovine rhinotracheitis infection. J. Am. Vet. Med. Assoc. 163:850-851.

193. Shortman, K., W. J. Byrd, J. C. Cerottini, and K. T. Brunner. 1973. Characterization and separation of mouse lymphocyte subpopulations responding to phytohemagglutinin and pokeweed mitogen. Cell. Immunol. 6:25-40.

194. Silber, R. H., and C. C. Porter. 1954. The determination of 17,21-dehydroxy-20-ketosteroids in urine and plasma. J. Biol. Chem. 210:923-932.

195. Smart, K. M., and E. D. Kilbourne. 1966. The influence of cortisone on experimental viral infections. VI. Inhibition by hydrocortisone on interferon synthesis in the chick embryo. J. Exp. Med. 123:299-307.

196. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. 6th ed. The Iowa State University Press, Ames, Iowa. 593 pp.

197. Solomon, J. A. 1965. The turning out syndrome - a hypothesis. Blood Horse 85:714.

198. Solomon, J. B. 1971. Foetal and neonatal immunology. North-Holland Publishing Co., Amsterdam. 381 pp.

199. Spink, W. W., McCullough, L. M. Hutching, and C. K. Mingle. 1954. A standardized antigen and agglutination technique for human brucellosis. Am. J. Clin. Pathol. 24:496-498.

200. Sprinkle, T. 1975. Diagnosis - equine rhino. Norden. News 50: 16-18.

201. Stabenfeldt, G. H., H. Setoford, C. E. Franti, and R. D. Schechter. 1976. Assessment of normal adrenal function in the dog. Calif. Vet. 30:15-19.

202. Sterzle, J., and A. M. Silverstein. 1967. Developmental aspects of immunity. Pages 337-459 in F. J. Dixon and R. G. White, eds. Advances in Immunology Vol. 6. Academic Press, New York.

203. Stone, S. S. 1970. Comparison of bovine serum and colostral antibody: Effect of colostral antibody on vaccination of calves for contagious bovine pleuro-pneumonia (CBPP). Immunology 18: 369-377.

204. Talone, G., L. Bonasera, A. Bellavia, and F. Ferina. 1974. Effects of cortisol on immune cytolysis. Path. Microbiol. 40: 30-36.

205. Targowski, S. P. 1975. Effect of prednisolone on the lymphocyte counts of ponies and on the reactivity of lymphocytes in vitro and in vivo. Infect. Immun. 11:252-256.

206. Tarr, M. J., and R. G. Olsen. 1978. Suppression of the cell-mediated immune system of the horse by systemic corticosteroid administration. J. Equine Med. Surg. 2:129-134.

207. Thomas, S. J., D. W. Wilson, C. G. Pierrepoint, E. H. D. Cameron, and K. Griffiths. 1976. Measurement of cortisol, cortisone, 11-deoxycortisol and corticosterone in foetal sheep plasma during the perinatal period. J. Endocrinol. 68:181-189.

208. Thompson, J., and R. Van Furth. 1973. The effect of gluco-corticosteroids on the kinetics of mononuclear phagocytosis. J. Exp. Med. 137:10-21.

209. Thomson, G. R., and J. A. Mumford. 1977. In vitro stimulation of foal lymphocytes with equid herpesvirus I. Res. Vet. Sci. 22: 347-352.

210. Thorn, G. W., P. H. Forsham, F. T. Garnot Prunty, and A. Gorman Hills. 1948. A test for adrenal cortical insufficiency: The response to pituitary adrenocorticotrophic hormone. J. Am. Med. Assoc. 137:1005-1009.

211. Tilden, R. L. 1977. A new advantageous approach to the direct radioimmunoassay of cortisol. Clin. Chem. 23:211-215.

212. Tlaskalová, M., J. Šterzle, P. Hájek, M. Pospíšil, I. Říha, H. Marvanová, V. Kamarýtová, L. Mandel, J. Kruml, and F. Kovářu. The development of antibody formation during embryonal and postnatal periods. Pages 767-785 in J. Šterzl and I. Říha, eds. Developmental aspects of antibody formation and structure, Vol. 2. Academia, Publishing House of the Czechoslovak Academy of Sciences, Prague.

213. Tobin, T. 1979. Pharmacology review: The corticosteroids. J. Equine. Med. Surg. 3:10-15.

214. Todd, A. C., W. R. McGee, Z. N. Wyant, and K. P. Hollingsworth. 1951. Studies on the hematology of the thoroughbred horse. V. Sucklings. Am. J. Vet. Res. 12:364-366.

215. Topel, D. G., and R. A. Merkal. 1966. Effect of exogenous goitrogens upon some physical and biochemical properties of porcine muscle and adrenal gland. J. Anim. Sci. 25:1154-1158.

216. Uhr, J. W. 1966. Delayed hypersensitivity. Physiol. Rev. 46:359-419.

217. Uhr, J. W., and J. B. Baumann. 1961. Antibody formation. I. The suppression of antibody formation by passively administered antibodies. J. Exp. Med. 113:935-957.

218. Uhr, J. W., and J. B. Baumann. 1961. Antibody formation. II. The specific anamestic antibody response. J. Exp. Med. 113: 959-970.

219. Venkataseshu, G. K., and V. L. Estergreen. 1970. Cortisol and corticosterone in bovine plasma and the effect of adrenocorticotrophin. J. Dairy Sci. 53:480-483.

220. Wagner, W. C., and S. L. Oxenreider. 1972. Adrenal function in the cow. Diurnal changes and the effects of lactation and neurohypophyseal hormones. J. Anim. Sci. 34:630-635.

221. Weiner, J., T. S. Cottrell, W. Margaretten, and D. Spiro. 1967. Electron microscopic study of steroid induced reticuloendothelial blockage. Am. J. Pathol. 50:187-201.

222. Westerfield, C., and W. W. Dimock. 1946. The pathology of equine virus abortion. J. Am. Vet. Med. Assoc. 109:101-111.

223. Weston, W. L., M. J. Mandel, G. E. Krueger, and H. N. Claman. 1972. Differential suppressive effect of hydrocortisone on lymphocytes and mononuclear macrophages in delayed hypersensitivity of guinea pigs. J. Invest. Dermatol. 59:345-348.

224. Weston, W. L., H. N. Claman, and G. G. Krueger. 1973. Site of action of cortisol in cellular immunity. J. Immunol. 110: 880-883.

225. Whipp, S. C., R. L. Wood, and N. C. Lyon. 1970. Diurnal variations in concentrations of hydrocortisone in plasma of swine. Am. J. Vet. Res. 31:2105-2107.

226. Wilks, C. R., and L. Coggins. 1976. Immunity to equine herpesvirus type 1 (rhinopneumonitis): *In vitro* lymphocyte response. Am. J. Vet. Res. 37:487-492.

227. Wilks, C. R., and L. Coggins. 1977. In vitro cytoxocity of serum and peripheral blood leukocytes for equine herpesvirus type I - infected target cells. Am. J. Vet. Res. 38:117-121.

228. Yu, D. T. Y., P. J. Clements, H. E. Paulus, J. B. Peter, J. Levy, and E. V. Barnett. 1974. Human lymphocyte subpopulations: Effect of corticosteroids. J. Clin. Invest. 53:565-571.

229. Zolovick, A., D. W. Upson, and Eleftheriou. 1966. Diurnal variation in plasma glucocorticosteroid levels in the horse (Equus caballus). Endocrinology 35:17-21.

230. Zurier, R. B., and G. Weissmann. 1973. Anti-immunologic and anti-inflammatory effects of steroid therapy. Med. Clin. N. Am. 57:1295-1307.

## VIII. ACKNOWLEDGMENTS

I wish to express my sincere appreciation to all those who gave of their time and knowledge to assist my endeavor in this project. The indispensable contributions of the following people deserve special thanks:

to Dr. Merlin L. Kaeberle for stimulating my interest in Veterinary Immunology and for providing counsel and guidance during the course of the project and many helpful suggestions in preparing this manuscript;

to Dr. Tracy L. Clark for his encouragement and ever ready assistance with many aspects of this project, especially the procurement of experimental animals and housing facilities;

to Dr. Wallace M. Wass who served as my major professor and offered helpful advice for the improvement of this manuscript;

to Dr. Lawrence E. Evans who served on my committee and advanced sound advice for performing the cortisol assays;

to Dr. James A. Roth for his valuable guidance with the statistical analysis and for the willing sacrifice of many hours of his time to assist with the sampling procedures and data interpretation;

to Mr. Ervin C. Johnson for his expert technical assistance and personal effort;

to Mr. Randy D. Hubbard and Mr. David L. Stanley for cheerfully assisting with the laboratory procedures and data processing;

to Dr. M. Gary I. Riley for examining the histological sections and aiding in their interpretation;

to the staff of the Veterinary Clinical Pathology section for evaluating blood samples and carefully preparing smears;

to Drs. Paul G. Eness, William J. Owen, Jerry P. Kunesh and Steven R. LeVan for their helpful cooperation and support;

to Mrs. Pat Gunnells for excellently typing this manuscript;

to Norden Laboratories Inc., for performing titer evaluations, for providing equine rhinopneumonitis virus preparations and for financially supporting part of this project; and

to the American Quarter Horse Association for providing additional funding.

This work could not have been completed without the sacrifice, reassuring support, love and understanding of my wife, Joni. She also willingly offered typing assistance for which I am deeply indebted. To her, I dedicate this thesis.