Age-related changes in lymphocyte subsets of Quarter Horse foals

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Objective—To characterize changes in lymphocyte subsets over time in foals from birth to 18 weeks of age, accounting for differences among individuals, and to determine the effect of overnight storage of blood samples on foal lymphocyte subset concentrations.

Animals—8 healthy Quarter Horse foals from birth to 18 weeks of age.

Procedure—Blood samples were collected longitudinally from birth to 18 weeks of age and a CBC performed on each sample. The samples were stained for lymphocyte markers, either immediately or after overnight storage and analyzed by flow cytometry.

Results—Total leukocytes, total lymphocytes, and the absolute concentrations of all lymphocyte subsets increased significantly with age. The proportions of B29A$^+$, CD21$^+$, and equine major histocompatability complex class-II molecule$^+$ lymphocytes increased significantly with age. The proportion of equine (Eq) CD5$^+$, EqCD8$^+$, and EqWC4$^+$ lymphocytes decreased significantly with age. Significant differences among foals were found with respect to initial concentrations with respect to initial concentrations, but not with respect to the rate of increase of the various subsets tested. Significant differences were not found in subset values when comparing blood samples stained on the day of collection or after overnight storage at room temperature (approx 21 C) or under refrigeration.

Conclusions and Clinical Relevance—These results are consistent with an increase in subset numbers and proportions over time, but with individual differences among foals. The observation of individual differences in subsets among foals suggests that there may be individual differences in susceptibility to infectious disease during the perinatal period. The absence of an effect of overnight storage makes field studies of lymphocyte subset concentrations more feasible. (*Am J Vet Res* 2002;63:531–537)

Foals are susceptible to a wide variety of infective agents, and identifying factors that contribute to susceptibility is a major goal in equine neonatology. Some study results[1,2] have documented the importance of humoral immunity for protection against many diseases in foals. However, although humoral immune responses are most effective in resistance to the extracellular phases of animal pathogens, cell-mediated immunity is critical for resistance to and recovery from intracellular pathogens.[1,2] Because of the difficulty in assessing cell-mediated immunity, particularly in a clinical setting, few studies have examined its status as a predictor of disease susceptibility in domestic animals.

Enumeration of CD4 lymphocytes, although not a functional measure of cell-mediated immunity, has proven to be a reliable predictor of susceptibility to many opportunistic intracellular pathogens in persons infected with the human immunodeficiency virus. Guidelines published by the US Public Health Service and Infectious Disease Society of America strongly recommend that human immunodeficiency virus-positive individuals with low CD4 lymphocyte concentrations be started on prophylactic antimicrobial treatment for specific agents.[3] For example, with CD4 lymphocyte concentrations < 200/µl, prophylaxis for *Mycobacterium avium* complex is indicated; with CD4 lymphocyte concentrations < 50/µl, prophylaxis for *Pneumocystis carinii* should be initiated. For other agents, suspected exposure in an individual with a low CD4 lymphocyte concentration is an indication to initiate prophylactic treatment.

The correlation of low CD4 lymphocyte concentrations with susceptibility to opportunistic infection in human immunodeficiency virus-positive individuals, in addition to the susceptibility of CD4 T-lymphocyte-deficient mice to a variety of infective agents,[4] led us to hypothesize that foal susceptibility to infection with intracellular opportunists such as *Rhodococcus equi* may be correlated with low concentrations of **equine** (**Eq**)CD4 lymphocytes at the time the foals are exposed to the agent. Flaminio et al[5] did not observe low EqCD4 lymphocyte concentrations in foals with active *R equi* infection. However, in that study, lymphocyte subsets were evaluated during the course of clinical pneumonia in foals, and the ongoing infection and immune response may have altered the dynamics of lymphocyte subpopulations. Furthermore, because *R equi* infections are most commonly acquired early in life,[6] it may be that the first few weeks are the more critical times to measure EqCD4 lymphocyte concentrations to determine their value for predicting susceptibility to infection.

The primary goal of the study presented here was to establish reference ranges for lymphocyte subsets in foals, using preparative and analytic methods that can be applied to immunophenotypic assessments in clini-

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cal studies or in clinical practice. To accomplish this goal, we analyzed major lymphocyte subsets in foals, using well-characterized and readily available reagents, specifically B-lymphocyte markers (B29A and human CD21), T-lymphocyte markers (EqCD5, EqCD4, and EqCD8), and a functional subset of equine T lymphocytes (EqWC4). We also analyzed the effect of overnight storage of blood samples on foal lymphocyte subset concentrations. Finally, we assessed and characterized the degree of variability among foals in their expression of lymphocyte subsets early in life and applied statistical models that were likely to reveal such differences.

Materials and Methods

Experimental animals and sample collection—Eight Quarter Horse foals were followed from birth through 18 weeks of age. Blood samples were collected aseptically into tubes containing EDTA (2 paired tubes of 5 ml each) during weeks 1, 2, 3, 4, 8, 13, and 18 of life. Complete blood counts were conducted on each blood sample, using an automated hematology analyzer.[a] An aliquot of the blood sample was stained immediately, and other aliquots were stored overnight, either at room temperature (approx 21 C) or refrigerated, and stained the following day. Early in the study, a paired heparinized blood sample was collected for comparison with some of the EDTA-anticoagulated samples. The experimental protocol was approved by the Texas A&M University Laboratory Animal Care Committee. All foals remained healthy throughout the course of the study.

Monoclonal antibodies—Monoclonal antibodies used in our study recognized **major histocompatibility complex (MHC)** class-I molecules (EqMHC-I, clone H58A[b]),[7] major histocompatibility complex MHC class-II molecules (EqMHC-II, clone TH14B[b]),[7] a marker for granulocytes and monocytes (EqGM1, clone DH59B[b]),[8] EqCD5 (clone HT23A[b]),[8] EqCD4 (clone HB61[b]),[8] EqCD8 α-chain (clone ETC142B[b]),[9] EqWC4 (clone HB65A[b]), a marker for equine B lymphocytes (clone B29A[b]),[8] and human CD21 (clone B-Ly4[c]).[d] A mixture of IgG1 and IgG2a isotype controls[e] was used for negative control staining.

The antibodies were diluted to optimal concentrations (determined by titrations in our laboratory) in **Dulbecco PBS solution[e] containing 5% (vol/vol) serum (DPBSS-5)** obtained from healthy adult Quarter Horses.

Immunofluorescence staining of whole blood—Leukocytes were stained, using a modification of the procedure of Stewart.[10] Briefly, EDTA-anticoagulated whole blood was washed twice in DPBSS-5 and incubated 10 minutes at room temperature (approx 21 C) with goat IgG at a final concentration of 100 µg/ml to block Fc receptor-mediated binding. Following incubation, a quantity of blood containing 5 to 10×10^5 leukocytes was added to 12 × 75-mm tubes containing aliquots of the monoclonal antibodies and incubated for 20 minutes on ice. The cells were washed once in DPBSS-5 and incubated on ice for 20 minutes with 50 µl of fluorescein-labeled goat antibodies to mouse IgG heavy and light chains[e] diluted 1:10 in DPBSS-5. The cells were then washed and the erythrocytes lysed with a commercial lysing solution.[f] The remaining leukocytes were washed twice and resuspended in 300 µl of formalin (2% in PBS solution).

Flow cytometry—Cells were analyzed on a flow cytometer[g] equipped with a 15-mW air-cooled argon laser, using acquisition software provided by the manufacturer.[h] Fluorescence emissions were collected through a 530/30-nm bandpass filter. List mode data were acquired on a minimum of 5,000 events defined by a light scatter gate set to delineate the lymphocyte population. Data analysis was performed with flow cytometric data analysis software,[i] using forward- and side-light scatter to gate on the lymphocyte population. The purity of the analysis gate was assessed by the percentage of EqMHC-I$^-$ events and the percentage of EqGM1$^+$ events (granulocytes and monocytes), according to the formula:

$$\text{Gate purity} = \%\ \text{EqMHC-I}^+\ \text{events} - \%\ \text{EqGM1}^+\ \text{events in the light scatter gate}$$

Samples for which the gate purity was < 90% or for which there were > 5% EqGM1$^+$ events in the lymphocyte gate were excluded from further analysis. Of the 56 samples drawn from the 8 foals, 2 samples were rejected for monocyte contamination of the lymphocyte gate. Two other blood samples could not be collected at the designated time, leaving 52 of an expected 56 samples for analysis in our study.

The proportion of lymphocytes expressing a particular marker was calculated according to the formula:

$$\text{Relative \%} = 100 \times \text{measured \%} \div \text{gate purity}$$

The absolute concentration of lymphocytes expressing a particular marker was obtained by multiplying the relative % by the total lymphocyte concentration.

Statistical analysis—Data were analyzed, using exploratory, descriptive, and inferential methods. Exploratory data analysis included examination of graphic displays of the lymphocyte subset data as a function of age for each foal. Descriptive methods included examination of summary statistics for various cell subsets by foal at various ages. For inferential analysis, linear mixed effects models[11] were fitted.[j] For the linear mixed-effect modeling, individual foal was considered as a random effect, and age was considered as a fixed effect. For each lymphocyte subset, change in either absolute concentration (eg, number of EqCD4 lymphocytes/ µl) or the proportion (eg, proportion of lymphocytes that were EqCD4 lymphocytes) with age was modeled, using a common slope for all foals but random intercepts. The validity of the assumption that a common slope for all foals was appropriate for a given covariate (eg, EqCD4 lymphocyte number) was tested by comparing likelihood ratio test statistics of a model with a common slope for all foals but different (random) intercepts for each foal with a model with differing (random) slopes and intercepts for each foal.[11]

Effects of storage condition (fresh, overnight refrigerated, or overnight at room temperature [approx 21 C]) also were assessed, using linear mixed effects models. Models that included effects of storage conditions were fitted for those cell marker characteristics that were significantly associated with changes in age. This approach allowed us to examine the effects of storage condition while accounting for effects of age.

Goodness-of-fit of models was assessed graphically, in the following ways: first, the fitted values were plotted against standardized residuals; these plots were examined for outliers and any evidence of a systematic pattern. Second, the validity of the assumption of the normality of the random effects and standard errors was assessed by plotting the residuals versus quantiles of the standard normal (ie, normal quantile-quantile plot) and the random effects versus quantiles of the standard normal. Finally, the fitted data were plotted against the observed data to visually assess agreement. For all statistical analysis, a significance level of $P \leq 0.05$ was used.

Results

The immunophenotypes of foals for EqCD4, EqCD8, EqCD5, B29A, CD21, EqMHC-II, and EqWC4 were determined (**Table 1**). The total of B (B29A$^+$) and

Table 1—Changes in number of leukocytes and number and percentage of lymphocyte subsets over time in 8 foals expressed by slopes of linear mixed effects modeling and descriptive statistics

	Age*										
	1 week (n = 7)		4 weeks (8)		8 weeks (8)		18 week (5)				
Subsets	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Slope	95% CI (slope)	*P* value
EqCD4 (/μl)	424	166–854	948	555–1,588	1,057	601–1,467	1,743	891–2,450	9.0	6.5, 11.4	< 0.001
EqCD4 (%)	37.1	32.6–41.7	47.0	29.6–60.5	43.5	36.3–50.0	43.2	38.9–48.3	0.001	–0.047, 0.050	0.954
EqCD8 (/μl)	201	26–301	317	175–603	341	115–510	477	308–681	1.8	0.8, 2.8	< 0.001
EqCD8 (%)	18.7	6.3–31.3	16.2	9.5–23.1	14.2	5.9–23.5	12.6	6.7–17.4	–0.05	–0.08, –0.02	< 0.001
EqCD4:EqCD8	2.6	1.2–6.4	3.3	1.3–5.0	3.7	1.8–8.4	4.0	2.2–7.2	0.01	>–0.01, 0.03	0.1020
EqCD5 (/μl)	887	354–1,594	1,565	963–3,225	1,599	877–2,488	2,623	1,526–3,686	12.0	7.9, 16.1	< 0.001
EqCD5 (%)	78.8	63.6–90.4	76.6	60.2–88.0	65.7	59.0–79.3	66.3	58.8–72.7	–0.13	–0.18, –0.07	< 0.001
B29A (/μl)	194	18–540	414	154–819	678	343–1,083	1,142	400–1,652	9.1	7.3, 11.0	< 0.0001
B29A (%)	15.0	4.3–26.4	20.2	11.6–29.6	28.0	15.8–37.0	27.8	18.0–35.8	0.15	0.10, 0.20	< 0.001
CD21 (/μl)	195	14–580	304	113–557	419	233–939	640	281–1103	5.0	3.7, 6.3	< 0.001
CD21 (%)	14.3	3.4–28.3	14.7	8.3–21.7	16.7	11.9–32.1	15.8	12.5–23.9	0.06	0.03, 0.09	< 0.001
EqMHC–II (/μl)	89	12–167	493	266–773	746	421–1,237	1,360	757–1,829	10.9	8.9, 13.0	< 0.001
EqMHC–II (%)	11.2	0.6–23.7	24.5	17.4–34.4	31.4	19.4–42.3	34.4	30.1–39.7	0.20	0.14, 0.25	< 0.001
EqWC4 (/μl)	79	26–207	123	53–244	118	45–202	156	114–200	0.8	0.4, 1.2	< 0.001
EqWC4 (%)	6.9	3.4–12.1	6.2	3.4–9.3	3.9	2.7–6.2	4.2	2.8–6.7	–0.01	–0.03, >–0.01	0.0289
Lymphocyte (/μl)	1,152	410–2,046	2,019	1,248–3,700	2,412	1,476–3,339	3,960	2,220–5,070	21.4	16.1, 26.7	< 0.001
Leukocyte (/μl)	7,000	3,800–11,700	7,990	6,000–10,000	9,200	6,900–15,900	9,480	7,200–13,000	22.7	10.6, 34.8	< 0.001

*1 week = 1 to 6 days old (mean = 3). 4 weeks = 22 to 27 days old (mean = 25). 8 weeks = 55 to 60 days old (mean = 58). 18 weeks = 115 to 120 days old (mean = 118). CI = Confidence interval.

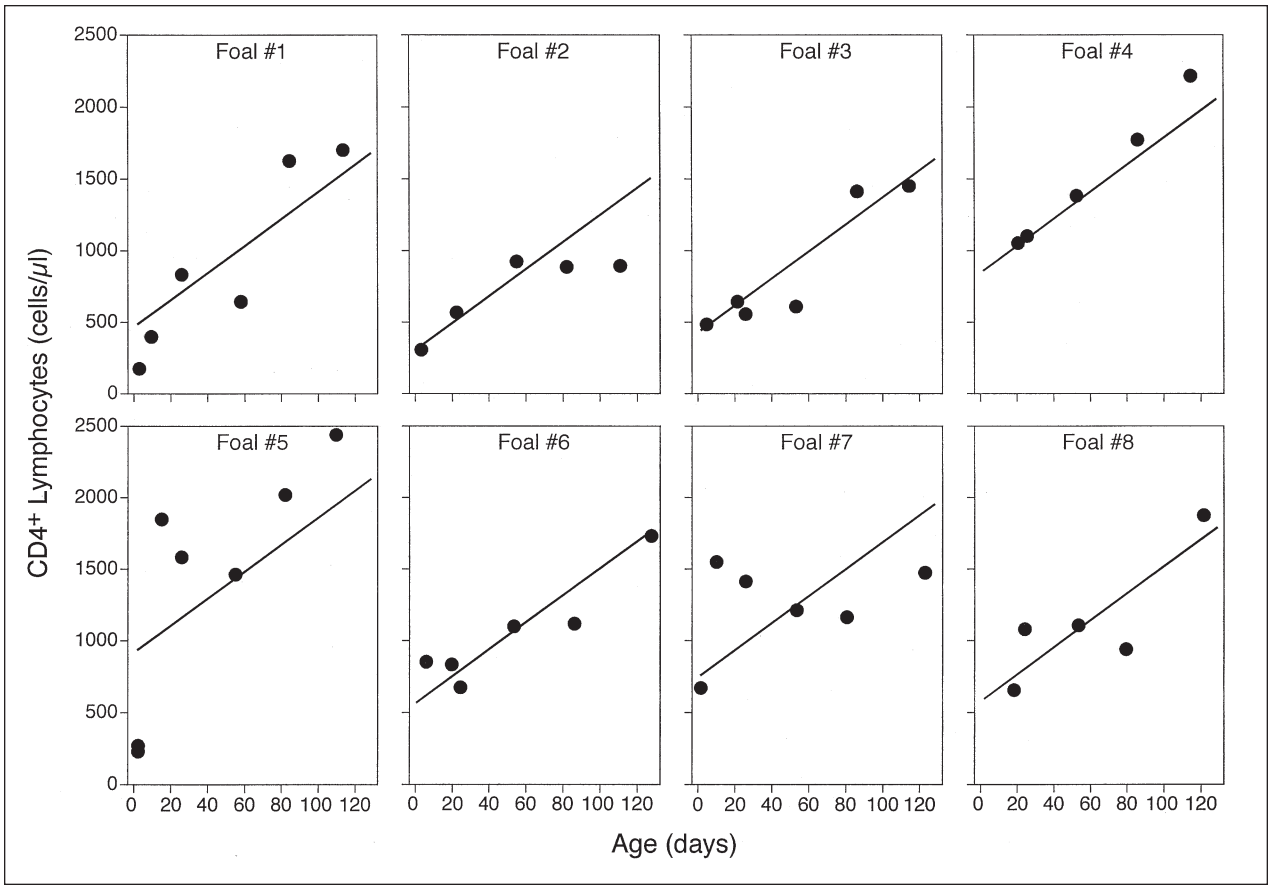

Figure 1—Linear plots of absolute concentration (cells/μl) of EqCD4+ lymphocytes over time. Leukocytes were stained and analyzed as described. Each panel represents the measured values (open circles) and modeled values (line) for a single foal.

T (EqCD5+) lymphocytes had a mean value of 96.0% (median, 97.0%; range, 76 to 109%), indicating that essentially all lymphocytes were accounted for by these markers. In 7 samples, the total of B and T lymphocytes was < 90%, possibly representing a natural killer cell population for which we did not test.[12]

The EqCD5+ T-lymphocyte population was incompletely accounted for by the sum of EqCD4+ and

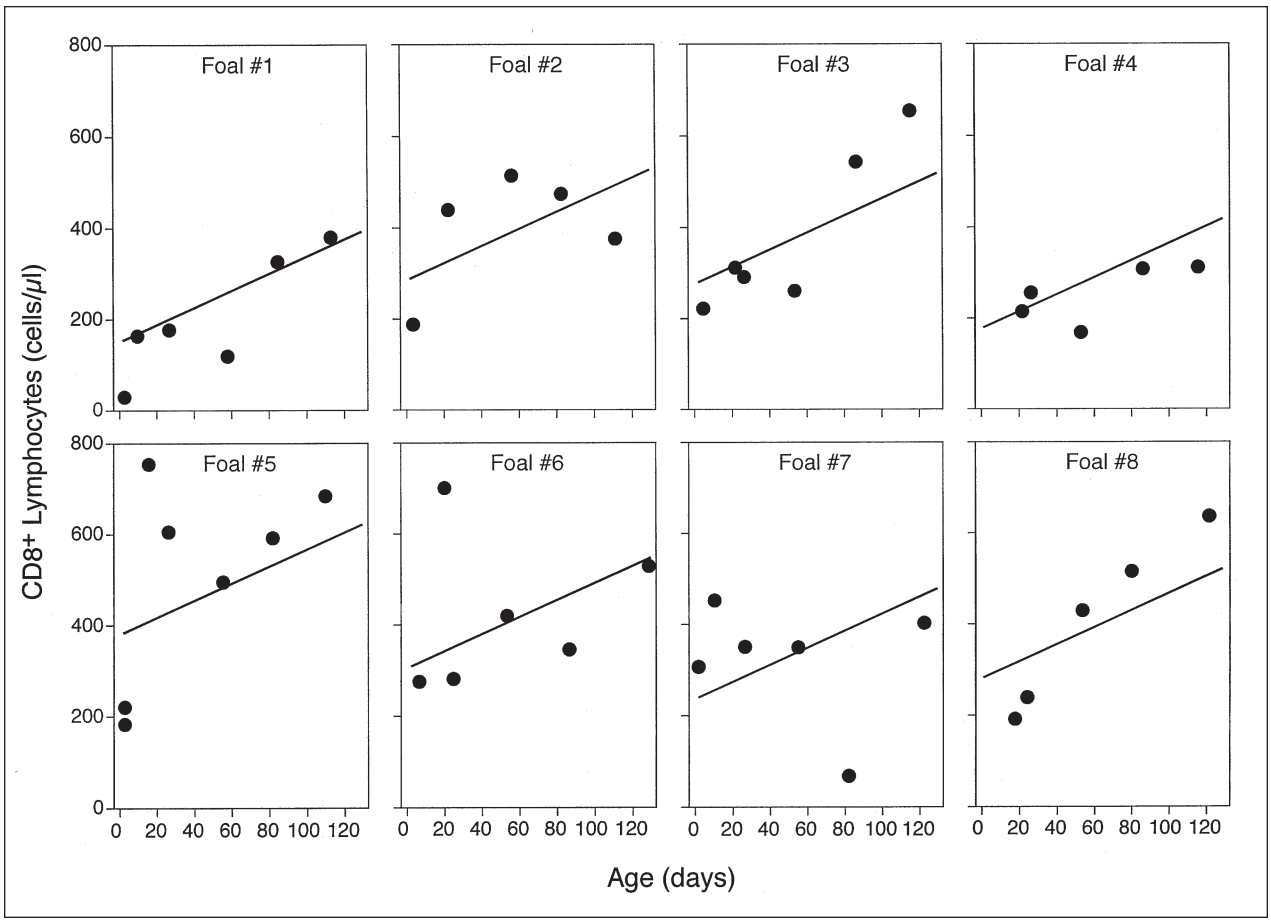

Figure 2—Linear plots of absolute concentration (cells/μl) of EqCD8$^+$ lymphocytes over time. Leukocytes were stained and analyzed as described. Each panel represents the measured values (open circles) and modeled values (line) for a single foal.

EqCD8$^+$ cells, with a mean excess of 14.6% EqCD5$^+$ lymphocytes (median, 12.4%; range, –2 to 39%). In 7 samples, the calculated number of EqCD5$^+$ EqCD4$^-$ EqCD8$^-$ lymphocytes was > 20%, indicating the likelihood of a population of double-negative T lymphocytes, possibly expressing the γδ T-cell receptor. Interestingly, 6 of the 7 samples with an excess of EqCD5$^+$ lymphocytes were in foals < 1 month old, with 5 being in foals < 9 days old (data not shown). Further, 3 of the 8 foals never exceeded an excess of 16% at any time.

Total leukocytes, total lymphocytes, and the absolute concentrations of all lymphocyte subsets measured increased significantly with age (Table 1). In addition, the proportions of B29A$^+$, CD21$^+$, and EqMHC-II$^+$ lymphocytes increased significantly with age. The proportion of EqCD5$^+$, EqCD8$^+$, and EqWC4$^+$ lymphocytes decreased significantly with age. A significant change with age was not found in the ratio of EqCD4$^+$ to EqCD8$^+$ lymphocytes, the number of segmented neutrophils, the number of monocytes, or the proportions of EqCD4$^+$ lymphocytes.

Evidence of significant random effects was found for each of covariate (ie, significant differences in intercepts or initial cell concentrations among foals). Further analysis of each of covariate revealed no significant difference between a model with random effects for both the slope and intercept versus a common slope for all foals and random effects for the intercept (ie, no significant difference between foals with respect to slope). Plots of EqCD4$^+$ and EqCD8$^+$ lymphocyte concentrations versus age (**Fig 1 and 2**) revealed that the number of EqCD4$^+$ and EqCD8$^+$ lymphocytes increased with age in a fairly linear manner and that there are clear, although not significant, differences in the slopes among foals (Fig 1 and 2).

For most samples in our study, an aliquot of blood was held overnight, either refrigerated or at room temperature (ie, approx 21 C), then stained and analyzed the following day. Although values for samples stored overnight at room temperature were often lower than those for either refrigerated overnight or fresh samples, the difference was not significant for any of the cell types after accounting for age and anticoagulant; plots of the EqCD4 staining by foal for those samples illustrate this finding (**Fig 3**). The light scatter gate for lymphocytes was not compromised by overnight storage, either refrigerated or at room temperature, as indicated by the absence of change in EqMHC-I$^+$ and EqGM1$^+$ events in the gate (data not shown). In addition, early in our study, some paired samples were collected in heparin and EDTA anticoagulants, and only minor differences were observed between them (data not shown).

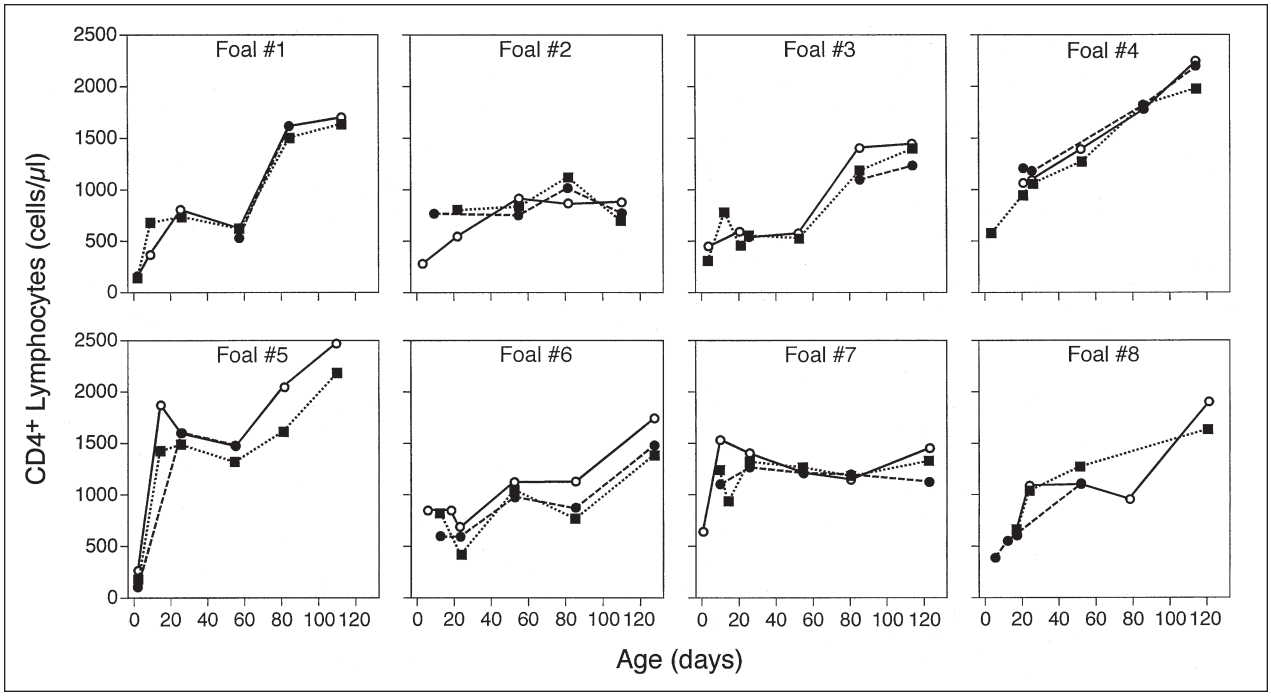

Figure 3—Evaluation of the effect of overnight storage on measurement of the absolute concentration (cells/μl) of EqCD4$^+$ lymphocytes. Leukocytes were stained and analyzed as described. Each panel represents the measured values with cells stained at the time of collection (open circles, solid line), after overnight storage in a refrigerator (closed squares, dotted line), and after overnight storage at room temperature (approx 21 C; closed circles, dashed line) for a single foal.

Discussion

The primary goal of our study was to establish reference ranges for lymphocyte subsets in foals, using preparative and analytic methods that can be applied to immunophenotypic assessments in clinical studies or in clinical practice. As such, we had 3 specific objectives. First, we analyzed all major lymphocyte subsets in foals, using well-characterized and readily available reagents, thus allowing comparisons to existing literature and extending existing knowledge by analyzing subsets not previously described for foals. Second, we analyzed the effect of overnight storage of blood samples on lymphocyte subset determination. Third, we assessed and characterized the degree of variability among foals in their expression of lymphocyte subsets early in life and applied statistical models to reveal such differences.

Specifically, results of our study demonstrate substantial agreement with previous studies of foal lymphocyte subsets, provide new information on subsets not previously characterized in foals, demonstrate differences among individual foals in age-related lymphocyte subsets, and reveal only minor effects of overnight storage or anticoagulant on the determination of lymphocyte subsets. Together, these results establish a basis for immunophenotyping foals in clinical studies.

We were especially interested in assessing and accounting for the degree of variability among foals in their expression of lymphocyte subsets early in life and applied statistical models that were likely to reveal such differences. With respect to values of intercepts (ie, initial concentrations) for each of the cell types, significant differences among foals were identified, indicating important differences among foals in age-specific values of lymphocyte subsets. A significant difference was not found among foals with respect to the rate of change of cell types with age (ie, the slope of subset numbers or proportions vs age); however, this lack of significance may have been attributable in part to the limited statistical power of studying only 8 foals. Visual inspection of our data indicated that the rate and pattern of change may be quite variable for some foals (Fig 1–3).

An underlying assumption of our analysis was a linear relationship in the change of subset numbers or proportions over time. Although this assumption did not always appear to fit all foals, using nonlinear modeling did not result in significant improvement of fit (data not shown), and graphic assessment of residual plots and observed-versus-fitted data indicated reasonably good fit of the linear models.

Our data on foal EqCD5$^+$, EqCD4$^+$, EqCD8$^+$, and B29A$^+$ lymphocyte subsets agree substantially with those of Flaminio et al.[5,13] In separate studies, that group analyzed these lymphocyte subsets in Quarter Horse and mixed-breed foals from birth to 4 or 6 months of age. Although we also found an age-dependent increase in our foals, the total lymphocyte concentration in our foal population was substantially lower than those in the previous studies. Reflecting the difference in total lymphocyte concentrations, the absolute concentrations of EqCD5$^+$, EqCD4$^+$, EqCD8$^+$, and B29A$^+$ lymphocyte subsets in our study were substantially lower than those reported by Flaminio et al. Another minor difference is that we found the proportion of EqCD8$^+$ cells decreased slightly over the first 18 weeks, whereas the previous studies reported no change over the first 4 to 6 months.

To characterize foal lymphocyte subsets even further, we included the monoclonal antibody B-Ly4, specific for human CD21, based on the manufacturer's catalog identification of its cross-reactivity with equine lymphocytes. The CD21 is expressed by mature B lymphocytes as well as follicular dendritic cells and is a membrane glycoprotein that forms part of the CD19-CD21-CD81 B-cell signaling complex.[14,15] In most all of our samples, fewer cells stained positive for CD21 than with B29A. This was also true for a small population of adult horses (data not shown), although the proportion of lymphocytes staining positive for either marker was small in the adults.

We also looked for age-related changes in a functional subpopulation of foal T lymphocytes. The EqWC4$^+$ lymphocytes represent a small subpopulation of EqCD5$^+$ cells that are reported to spontaneously transcribe interleukin-2 and interferon-γ in vitro.[16] On mitogen stimulation, transcripts for interleukin-2 decline, and those for interferon-γ increase; interleukin-4 transcripts can only be detected in mitogen-stimulated cells. Although these cells seem to be activated, expression of EqWC4 did not increase on stimulation, indicating that it is not an activation marker. The EqWC4 molecular weight suggests that it may be an equine homologue of CD28, but its cellular expression pattern argues against that conclusion.[16,17] We included EqWC4 in our study because of the possibility that the numbers of circulating activated T lymphocytes may increase with age. That, however, was not our observation; although there was a small but significant increase in the absolute concentration of EqWC4$^+$ lymphocytes over the course of our study, the proportion of EqWC4$^+$ lymphocytes declined.

Because a goal of our study was to develop an immunophenotyping panel that could be used in clinical studies of foals, it was important to use measures that assessed the completeness of our immunophenotyping panel. First, the purity of the lymphocyte gate was assessed by the percentage of EqMHC-I$^-$ and EqGM1$^+$ events in the gating region. Using those criteria, we could calculate a corrected percentage of positive lymphocytes for each subset. Second, we assessed the immunophenotyping by determining the extent of the total lymphocyte population that was accounted for by the sum of T- and B-lymphocyte subsets and the extent of T-lymphocyte population that was accounted for by the sum of CD4 and CD8 subsets.

In accounting for total lymphocytes, we found that the sum of the B29A$^+$ (B lymphocytes) and EqCD5$^+$ (T lymphocytes) subsets accounted for all but a small percentage of lymphocytes. Our results are similar to others that have reported such values, mostly in adult horses.[18-21] All but 1 of those studies[20] used surface **immunoglobulin (sIg)** expression to enumerate B lymphocytes. We chose not to use sIg expression, because our experience in a variety of species is that the amount of expression of sIg is often heterogeneous, with no distinct population of sIg$^+$ cells that can be reliably separated from sIg$^-$ cells.

A concern with using EqCD5 as the T lymphocyte marker is the potential for detecting EqCD5$^+$ B lymphocytes. Although such cells have been found in horses,[18] other investigators have not been able to detect such cells. In both the first and second workshops on equine leukocyte antigens, it was reported that no laboratories were able to detect EqCD5$^+$ B lymphocytes.[8,17] It would be valuable to use EqCD3 as the marker for T lymphocytes; however, the single monoclonal antibody recognizing EqCD3 is not commercially available, limiting its applicability to clinical assessments. One commercially available monoclonal antibody, raised against a synthetic peptide of human CD3, cross-reacts with equine lymphocytes but recognizes an intracellular epitope. The requirement that cells be permeabilized to use this antibody would limit its application in routine clinical studies. In published comparisons of monoclonal antibodies to EqCD3 and EqCD5, nearly identical percentages of leukocytes were labeled by each antibody.[17,21]

Presumably the small fraction of non-T, non-B lymphocytes in the foal blood samples were natural killer cells. Although 1 monoclonal antibody that recognizes equine natural killer cells has been reported,[12] it is not commercially available, thus limiting its potential usefulness for clinical assessments of foals.

The accounting for all T lymphocytes was less complete, as the sum of EqCD4$^+$ and EqCD8$^+$ lymphocytes averaged 14% fewer than the total EqCD5$^+$ lymphocytes. Although their data were reported in graphic rather than tabular form, the studies of Flaminio et al[5,13] appear to find a similar discrepancy in the foals they analyzed. Similarly, Lunn et al[19] reported a discrepancy of about 13% in the mean value for 5 adult horses and a range of 2 to 10% in 3 individual adults. In a study of blood preparative techniques, comparing analysis of total leukocytes to density gradient-separated mononuclear cells, Akens et al[22] reported smaller differences of –1 to 5%.

Perhaps the most likely explanation is that, particularly in foals, there is a substantial subset of lymphocytes expressing the γδ T-cell receptor. As already mentioned, the possibility must be considered that some of these EqCD5$^+$ EqCD4$^-$ EqCD8$^-$ cells are EqCD5$^+$ B lymphocytes, although others have been unable to detect such cells and EqCD3, and EqCD5 appear to be expressed on the same lymphocyte populations.

It is interesting to observe that this discrepancy was more pronounced in the first few weeks of life, suggesting the possibility that γδ T lymphocytes may be more prevalent in young foals. We further observed that 3 of the 8 foals in our study never had an excessively high number of EqCD5 lymphocytes expressing neither EqCD4 nor EqCD8. Although few previous studies reported individual variation among test animals, Lunn et al[19] did report 3 individual adults in which the EqCD4 and EqCD8 accounted for only 82, 84, and 86% of lymphocytes (by dual staining), whereas EqCD5 labeled 94, 91, and 88% of lymphocytes, respectively. Thus, it will be of interest to characterize this population of cells as reagents become available to do so and especially to determine individual variation in the frequency of the EqCD5$^+$ EqCD4$^-$ EqCD8$^-$ cells.

We found only minor insignificant differences in subset values when comparing blood samples collected in heparin or EDTA and when comparing blood sam-

ples stained on the day of collection or after overnight storage at room temperature (approx 21 C) or under refrigeration. The absence of effect of overnight storage makes it practical to conduct field studies and clinical assessment of foals, because it is often necessary to ship samples from distant sites. In particular, it does not appear that refrigeration is necessary to preserve lymphocyte subsets in samples stored overnight.

The immune competency of foals is of considerable interest in equine medicine. In our study, we have measured changes in the representation of major lymphocyte subsets over the first 18 weeks of life in 8 foals. Our results are consistent with a linear increase in subset numbers and proportions during that period. Significant differences were found among foals with respect to the values of intercepts (ie, initial concentrations) but not with respect to the rate of increase of the various subsets tested. The lower intercepts for lymphocyte subsets, and particularly for CD4 lymphocytes, in certain foals may enhance their susceptibility to infectious diseases during the perinatal period. Results of our study provide a baseline from which to analyze foal lymphocyte populations in clinical studies.

[a]Cell-Dyne 5400 hematology analyzer, Abbott Laboratories, Chicago, Ill.
[b]VMRD Inc, Pullman, Wash.
[c]BD Pharmingen, San Diego, Calif.
[d]Research Products Catalog, BD Biosciences, San Diego, Calif. and J. Yu, Personal communication.
[e]Sigma Chemical Co, St Louis, Mo.
[f]FACSLyse, Becton Dickinson Immunocytometry Systems, San Jose, Calif.
[g]FACSCalibur, Becton Dickinson Immunocytometry Systems, San Jose, Calif.
[h]Cell Quest, Becton Dickinson Immunocytometry Systems, San Jose, Calif.
[i]FlowJo, TreeStar, Palo Alto, Calif.
[j]S-PLUS 2000, Mathsoft Inc, Seattle, Wash.

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