

Age-related quantitative alterations in lymphocyte subsets and immunoglobulin isotypes in healthy horses

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Objective—To characterize age-associated changes in lymphocyte population subsets and immunoglobulin isotypes.

Animals—30 healthy young light-breed horses (5 to 12 years old) and 30 healthy aged light-breed horses (> 20 years old).

Procedure—Lymphocyte subset populations were identified, using monoclonal antibodies to cell surface markers CD5, CD4, CD8, and IgG. Subset populations were quantitated by use of flow cytometric analysis of antibody-stained cells. Serum immunoglobulin concentration was determined using single radial immunodiffusion.

Results—Absolute cell counts of total lymphocytes, T cells, CD4⁺ and CD8⁺ T cells, and B cells were decreased in aged horses, compared with young horses. There was a significant decrease in the percentage of CD8⁺ cells and an increase in the CD4⁺-to-CD8⁺ cell ratio in the aged population, compared with young horses. However, serum concentration of IgG, IgG(T), IgM, or IgA did not differ with age.

Conclusions and Clinical Relevance—In horses, total lymphocyte count and lymphocyte subset cell counts decrease with age. Age-matched control values are necessary for optimal evaluation of hematologic variables in aged horses. The decrease in lymphocyte subset cell counts in healthy aged horses mimics that seen in other species and may contribute to an age-associated decrease in immunocompetency. (*Am J Vet Res* 2001;62:1413–1417)

Age-related changes in humoral and cell-mediated immunity have been suggested to contribute to a decrease in immune competency in aged individuals. A decrease in immune function may increase infectious morbidity in aged populations. This age-related decrease, termed immunosenescence, has been evaluated in humans, dogs, and rodents.¹⁻³ Several common features have been identified in all species. The decrease in function chronologically parallels the involution of the thymus.^{4,5} T-helper cell activity is substantially altered, resulting from changes in function of the cells as well as shifts in lymphocyte subset populations.⁴ Immunologic markers of chronic inflammation increase with age.⁶ The incidence of autoimmune dis-

ease and neoplasia is greater in geriatric populations, compared with young populations.^{5,6}

The population of geriatric horses (ie, those 20 years or older) has increased substantially in the past decade. It is estimated that geriatric horses constitute 10 to 20% of the total equine population.⁷⁻⁹ This demographic shift may represent a change in the horse's role in society to one of a companion animal. In our hospital, there was a 55% increase in the number of horses 20 years or older between 1990 and 1995. Despite this growing population, minimal information is available about normal age-associated physiologic events in horses.

In a previous study from our laboratory, aged horses were determined to have a significant decrease in total peripheral lymphocyte count.¹⁰ The objective of the study reported here was to further define this age-associated change by comparing lymphocyte population subsets and immunoglobulin isotypes between young and aged horses.

Methods and Materials

Study population—Horses used for the present study were selected and determined to be healthy as described.¹⁰ Thirty horses 20 years of age or older were identified from the field service records of the North Carolina State University Veterinary Teaching Hospital; these horses served as the aged or geriatric group. Age was determined by registration (18 horses), single ownership for > 25 years or since the horse was ≤ 4 years of age (8 horses), or history provided by current owner (4 horses). Horses were determined to be healthy on the basis of veterinary records, history as reported by the owner, thorough physical examination, CBC results, and serum biochemical analyses. Evidence of illness or trauma in the previous 6 months, history of recent weight loss, or evidence of an abnormal coat or shedding pattern excluded the horse from the study. Ponies and draft horses were not included. Thirty horses age 5 to 12 years were selected as young control horses on the basis of the same criteria. Whenever possible, a young control horse was selected from the same environment as a geriatric horse.

Analysis of lymphocyte subset distribution—Analysis of lymphocyte subsets was performed as described.¹¹ Briefly, blood was obtained by external jugular venipuncture and collected into 10-ml evacuated tubes containing EDTA.^a At the same time as lymphocyte subtyping, a sample was submitted for CBC. White blood cell counts were determined, using an electronic cell counter calibrated for equine blood.^b Differential WBC counts were determined manually by a clinical pathology technician. The total lymphocyte count was used to calculate absolute lymphocyte subset counts for each horse. Blood was centrifuged for 3 minutes at 120 × g, and the plasma layer containing WBC was transferred to 50-ml polypropylene conical tubes with 2 to 2.5 ml of plasma/tube. Forty milliliters of lysis solution (8.29 g of NH₃Cl and 1 g of KHCO₃ dissolved in 1 L of distilled water) was added to each

Received Oct 2, 2000.

Accepted Nov 16, 2000.

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conical tube, gently mixed with the plasma, and incubated at room temperature (approx 25 C) for 10 minutes with intermittent gentle mixing. Tubes were centrifuged for 10 minutes at 1,000 X g, and the supernatant was discarded. Cell pellets were washed 3 times, using RPMI media with 0.4% fetal bovine serum (FBS). The cell pellet was resuspended in RPMI with 0.4% FBS, and cells were counted.

For T-cell analysis, WBC were aliquoted to 1.5-ml microcentrifuge tubes at a final count of 1×10^6 cells/tube, pelleted in a microcentrifuge, and resuspended in a 1:100 dilution of the appropriate monoclonal antibody against equine CD5 (a pan-T cell marker), CD4, or CD8.^c These antibodies have been characterized.¹² The secondary antibody, fluorescein isothiocyanate- (FITC-) conjugated goat anti-mouse IgG and IgM,^d was then added to samples for detection of cells with bound primary antibody. After washing, surface fluorescence was quantitated by use of flow cytometry.^e

For B-cell analysis, washed WBC were resuspended in PBS solution with 0.4% FBS, incubated in the dark without added antibody at 4 C for 30 minutes, and washed 3 times in PBS solution with 0.4% FBS. Washed cells were resuspended in a 1:500 dilution of an affinity-pure F(ab')₂ fragment of goat anti-equine IgG and IgM (heavy and light chain) conjugated to FITC and incubated for 30 minutes at 4 C. After washing, surface fluorescence was quantitated by use of flow cytometry.

Lymphocyte subset counts were determined by flow cytometric analysis of antibody-stained cells. Lymphocytes were identified by analysis of forward- and side-angle scatter and gated to exclude neutrophils from the analysis. For each sample, 10,000 gated events were counted. A dot plot of forward scatter versus fluorescence (channel 1) was generated and analyzed, using the instrument software.^f

Determination of immunoglobulin concentration—

Serum immunoglobulin concentration was measured by use of a commercial single radial immunodiffusion kit.^g Blood was obtained from the external jugular vein by venipuncture and collected into evacuated glass tubes^h without anticoagulant. Blood was allowed to coagulate, and serum was stored at -70 C until assayed but not more than 6 months. Concentrations of IgM, IgG, IgG(T), and IgA were determined, using 3 μ l of thawed serum. Reference standards provided in the kit were used to calculate a linear regression line that was then used to determine concentrations in each sample.

Statistical analyses—Distribution of each data set was determined by use of the Kolmogorov-Smirnov test (with a Lilliefors correction). Normally distributed data were analyzed by use of a Student *t*-test. Data sets that were not normally distributed were analyzed by use of the Mann-Whitney rank sum test. In all instances, values for aged horses were compared with values for control horses. Linear regression analysis was performed on data, using age as the independent variable. Values of *P* < 0.05 were considered significant. A statistical software program^h was used to perform all analyses.

Results

The aged population included 12 mares, 17 geldings, and 1 stallion with a mean age of 23.3 years (range, 20 to 39 years; only 1 horse was > 30 years old). The control horse population consisted of 15 mares and 15 geldings with a mean age of 9.1 years. Both populations consisted of a mix of light-breed horses.

Absolute cell counts of total lymphocytes, CD5⁺, CD4⁺, and CD8⁺ lymphocytes, and B cells were significantly (*P* < 0.001) less in aged horses than in young control horses (Fig 1). Percentages of CD4⁺, CD8⁺, and CD5⁺ lymphocytes and B cells were also compared between groups by use of the Mann-Whitney rank sum

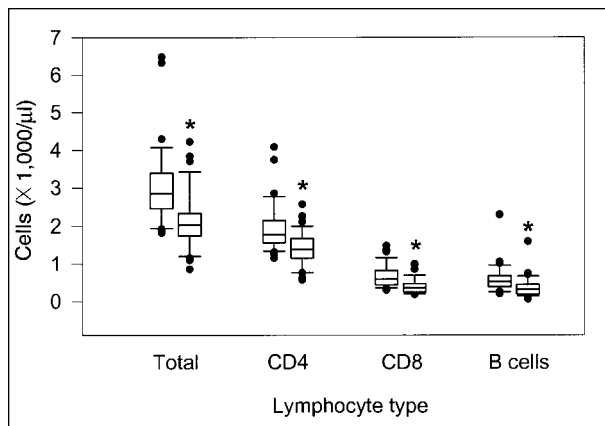


Figure 1—Lymphocyte counts in young horses (open boxes; 5 to 12 years old) and aged horses (shaded boxes; > 20 years old). Boxes represent median and 25th and 75th percentiles, and error bars indicate 10th and 90th percentiles for each designated population. *Significantly (*P* < 0.05) different between young and aged horses.

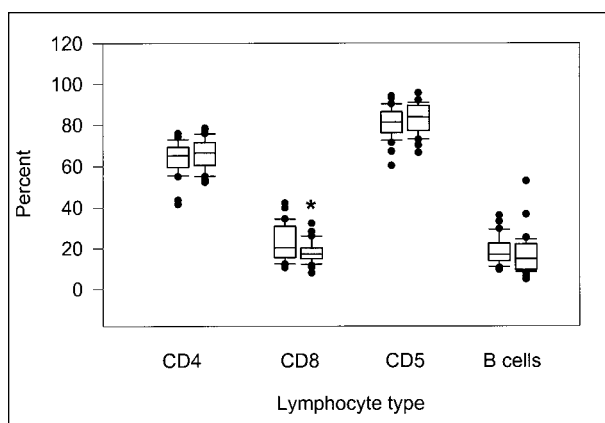


Figure 2—Percentage of peripheral blood lymphocytes in young horses (open boxes) and aged horses (shaded boxes). Boxes represent median and 25th and 75th percentiles, and error bars indicate 10th and 90th percentiles for each designated population. Boxes to the far right (plain and hatched) indicate the CD4⁺-to-CD8⁺ cell count (CD4/CD8) ratios in young and aged horses, respectively. *Significantly (*P* < 0.05) different between young and aged horses.

test (Fig 2). There was a significant (*P* = 0.039) decrease in the percentage of CD8⁺ cells in the aged horses, compared with young horses. The CD4⁺-to-CD8⁺ cell (CD4/CD8) ratio was calculated for each group, and results were compared between groups by use of a Student *t*-test. Mean (\pm SD) CD4:CD8 for aged horses was 4.07 ± 1.54 ; for young horses, it was 3.30 ± 1.31 . This difference was significant (*P* = 0.043).

Linear regression analysis of absolute counts of total lymphocytes and CD8⁺ cells and CD4:CD8 of all horses was performed, using age as an independent variable (Fig 3). Regression coefficients were 0.405 (*P* = 0.002), 0.318 (*P* = 0.014), and 0.500 (*P* < 0.001) for total lymphocyte count, absolute CD8⁺, and CD4:CD8 respectively. Data for regression analyses were not normally distributed because of the lack of horses in the 12 to 20 year age range.

Serum concentrations of immunoglobulin subtypes IgG(T), IgG, IgM, and IgA did not significantly differ between aged and young horses (Table 1).

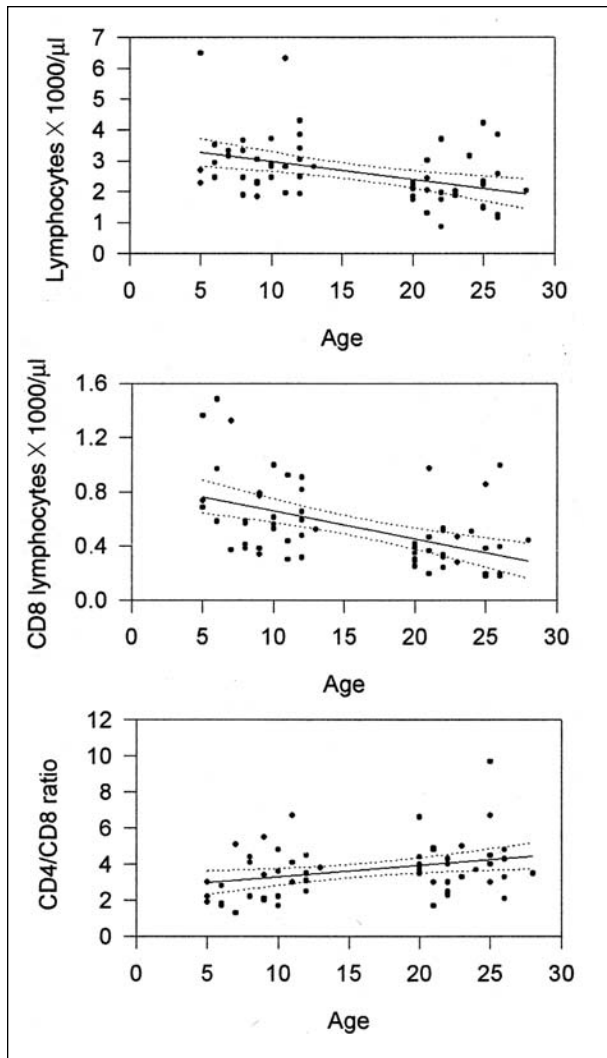


Figure 3—Linear regression analysis of total lymphocyte counts (top), absolute CD8⁺ cell counts (middle), and CD4/CD8 ratios (bottom) versus age. Data from one 39-year-old horse was deleted prior to regression analysis. Dotted lines represent 95% confidence intervals.

Table 1—Mean \pm SEM serum immunoglobulin concentrations (mg/dl) in 30 aged (> 20 years old) and 30 young (5 to 12 years old) healthy horses

Immunoglobulins	Horses		P values
	Aged	Young	
IgG	1,521 \pm 29.6	1,447 \pm 26.3	0.07
IgG(T)	689 \pm 33.7	746 \pm 39.5	0.28
IgM	191 \pm 8.3	75 \pm 8.2	0.17
IgA	299 \pm 13.4	261 \pm 14.9	0.07

Discussion

The immune system is a biological defense mechanism composed of 3 branches: nonspecific or innate immunity, antibody-mediated responses, and cell-mediated responses. The alteration of these responses that accompany aging is known as immunosenescence. These derangements of the immune system may contribute and potentially predict morbidity and mortality in aged populations.¹³ Identification of normal changes of the immune system in geriatric horses is a prerequisite

site for development of medical intervention protocols for disorders characterized by physiologic and pathologic age-associated immune alterations.

Studies in humans and laboratory animals have revealed immunosenescence to be a complex process involving dysregulation of all 3 immune branches. Evidence as to the nature of these changes is conflicting. The CD4:CD8 in human peripheral blood is reported by some to increase with age⁵ but by others to decrease with age.¹⁴ Similarly, serum IgG concentration increases¹⁵ or is unaffected¹⁴ by advancing age. Interferon gamma production increases,¹⁶ decreases,¹⁷ or remains unchanged with age.¹⁸

Conflicting results may be attributable to differences in experimental design, including the lack of in vitro assays to adequately simulate in vivo processes. Differences between species (humans vs rodents) studied may also influence results. Other factors, including diet, environmental stress, exercise, or hormonal profile, may impact immunoresponsiveness. For example, mild zinc deficiency in elderly humans reduces interleukin-2 (IL-2) production, lymphocyte response to mitogens, and decreases the peripheral CD4⁺ T-cell lymphocyte count.¹⁹ To minimize the effect of environment in our study, a young control horse was selected from the same farm as each geriatric horse.

Conflicting results regarding immunosenescence may also be attributable to difficulty in selection of healthy, aged subjects for study. This problem has been addressed in studies of human aging by establishing strict selection criteria, which requires results of clinical and laboratory tests be within reference range.²⁰ In our study, admission criteria included normal physical examination findings and CBC and serum biochemical analysis results within reference range. Particular attention was paid to subtle clinical indications of pituitary pars intermedia dysfunction, as this condition is common in geriatric horses and could potentially result in marked alterations in immune function of affected animals.

The definition of aged selected by the researcher will impact the outcome of the study as well. Healthy humans 100 years or older have decreased peripheral total lymphocyte, T-cell, and CD4⁺ and CD8⁺ cell counts, compared with humans 60 years of age.²¹ In our study, geriatric was defined as \geq 20 years old. This age was selected to be compatible with that used in previous reports^{10,22,23} but may not be biologically optimal.

Despite these described conflicting results, there are consistent age-associated changes to the immune system. T-cell mediated immune responses, including IL-2 production, IL-2 receptor production, delayed-type hypersensitivity, and responses to mitogen stimulation, are decreased in aged humans and rodents.^{2,18,24-26} The exact molecular mechanism of these changes remains speculative. Available data suggest age-related impairment of signal transduction pathways, such as calcium mobilization, kinase activity, and protein phosphorylation, contributes to T-cell dysregulation.^{1,25,27}

Humoral response to antigenic challenge also decreases with age, most noticeably in response to neoantigen.¹ However, it is unclear to what extent this

results from a decrease in B-cell responsiveness. Important decreases in humoral responses may be attributed to the decrease in CD4⁺ helper cell function.

A rearrangement of the distribution of lymphocyte populations is consistently found in geriatric patients; naive T cells decrease in number and memory T cells increase, likely as a result of thymic involution.²¹ Absolute numbers of T cells, T helper cells (CD4⁺), and T cytotoxic cells (CD8⁺) decrease.²¹ Total lymphocyte count and B-cell count have been reported to decrease^{21,26} or remain unchanged.¹⁶ In our study, a significant decrease in absolute numbers of peripheral lymphocytes, T cells, and B cells was observed in healthy geriatric horses (Fig 1). Within the T-cell population, T-helper cell (CD4⁺) and T-cytotoxic cell (CD8⁺) numbers were significantly decreased. For CD4⁺ cells, this was caused solely by a decrease in absolute numbers of cells; for CD8⁺ cells, it was a decrease in numbers and percentage.

The CD4:CD8 is a nonspecific indicator of inflammation or immunodeficiency. Increases in the CD4:CD8 may accompany inflammatory diseases such as inflammatory bowel disease or granulomatous pneumonia.^{28,29} Decreases in the CD4:CD8 result from depletion of T-helper cells in immunosuppressive conditions such as feline or human immunodeficiency virus infection.^{30,31} Reports of changes in the CD4:CD8 in geriatric humans and rodents are conflicting.^{5,14} In our study, geriatric horses had an increased CD4:CD8, compared with young control animals. This may suggest a predominantly inflammatory state in geriatric horses. Other markers of a proinflammatory state include increased concentrations of positive acute phase proteins, decreased concentrations of negative acute phase proteins, and increased activity of inflammatory cytokines. Serum concentration of fibrinogen, a positive acute phase protein, and albumin, a negative acute phase protein, are unchanged in aged horses.¹⁰ To our knowledge, other acute phase proteins and inflammatory cytokines have not been assessed in geriatric horses. Therefore, the importance of the increase in the CD4:CD8 observed in aged horses remains speculative.

Serum concentrations of immunoglobulins in aged horses (IgM, IgG, IgG[T], and immunoglobulin A) were not significantly different from that in young horses (Table 1). In humans, IgG and IgA concentrations increase with age, whereas IgM concentration remains unchanged.³² In mice, multiple isotype immunoglobulin concentrations were shown to increase 2- to 6-fold with age.¹⁵

Identification of the immune alterations that accompany normal physiologic aging in horses will assist in development of more appropriate preventative health programs. Differences in the ability of geriatric horses to respond to antigenic stimulation may also affect vaccination strategies. A possibility of an increased risk for development of inflammatory conditions suggests that prophylactic use of anti-inflammatory agents such as vitamin E, thiamine, or glycosaminoglycan derivatives may be beneficial in older horses. Further characterization of the impact of dietary constituents such as zinc on immunosenescence in horses will assist in future recommendations for feeding this population. To our knowledge, this is the first report of

the age-related immune changes observed in healthy geriatric horses. Additional studies are necessary to further define the functional immune changes expected with age to allow veterinarians to optimize health management strategies for older horses.

^aVacutainer system, Becton Dickinson, Rutherford, NJ.

^bSeronoBaker 9010+ hematology analyzer, SeronoBaker, Allentown, Pa.

^cCourtesy of Lunn DP, University of Wisconsin, Madison, Wi.

^dJackson Immunoresearch, City, Pa.

^eFACScan, Becton Dickinson Immunocytometry Systems, San Jose, Calif.

^fLysis II, Becton Dickinson Immunocytometry Systems, San Jose, Calif.

^gSRID Kit, VMRD Inc, ProScience Corp, Pullman, Wash.

^hSigmaStat statistical software, Jandel Scientific, San Rafael, Calif.

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