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UTILITY OF TUMOR NECROSIS FACTOR-α AND INTERLEUKIN-6 AS PREDICTORS OF NEONATAL MORTALITY IN WHITE-TAILED DEER

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Blood serum was collected between June 1990 and August 1992 from newborn white-tailed deer (*Odocoileus virginianus*) fitted with radiocollars. We measured serum concentrations of immunoreactive tumor necrosis factor-α (iTNF-α) and immunoreactive interleukin-6 (iIL-6) to relate cytokine expression to probability of mortality during the first 21 days of life. Stepwise logistic regression indicated that iTNF-α, hemolytic complement, gamma globulins, gamma glutamyl transferase, and mass/length^3 could predict survival of white-tailed deer during the first 21 days of life with 90.9% accuracy. Univariate logistic regression did not show a relationship between serum concentrations of iTNF-α or iIL-6 and probability of mortality. However, fawns that died before 21 days of age tended to have greater levels of iTNF-α (688.4 ± 168.8 pg/ml) than survivors (412.9 ± 81.2 pg/ml). Although these data suggest that iTNF-α may be a useful predictor of stress, additional research is needed to understand response of cytokines to neonatal stress and mortality and to elucidate their utility as indices.

Key words: cytokine, immunocompetence, interleukin-6, mortality, neonatal, *Odocoileus virginianus*, tumor necrosis factor-α, white-tailed deer

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Cytokines are regulatory proteins secreted by T-cells that mediate cellular interactions during immune responses. These compounds are an integral part in the recognition of and response to diseases, parasites, and other foreign bodies. Tumor necrosis factor-α (TNF-α) is a cytokine associated with antitumor activity, lysis of virally infected cells, inflammatory responses, and production of interleukin-6 (IL-6—Beutler and Cerami 1989; Spaulding et al. 1997). IL-6 influences immune responses such as immunoglobulin release by B-cells, differentiation of cytotoxic T-cells, and inflammatory responses (Spaulding et al. 1997; Tizard 1992).

Our understanding of TNF-α and IL-6 in the immune response of laboratory species and humans suggests that these cytokines could be used to assess effects of environmental stressors on wildlife populations. Duffy et al. (1994a, 1994b) reported significantly elevated levels of IL-6 in river otters (*Lutra canadensis*) from oil-polluted waters of Prince William Sound, Alaska, after the *Exxon Valdez* oil spill in 1989, which they attributed to chronic physiologic stress. Spaulding et al. (1997) found that concentrations of circulating IL-6 dropped in laboratory mice (*Mus*) under a 50% caloric re-

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striction. Although these studies indicate that concentrations of circulating cytokines are influenced by environmental stressors, our understanding of relationships between cytokines and environmental stressors (i.e., poor nutrition, toxins, disease) is incomplete.

We assessed the feasibility of using levels of TNF-α and IL-6 in blood serum as predictors of neonatal mortality in a high-density population of white-tailed deer (*Odocoileus virginianus*). Sams et al. (1996b) found that altered immunocompetence was a major factor in mortality of neonates from a high-density population of white-tailed deer because of poor maternal nutrition and subsequent breakdown in passive transfer of immunity from mother to fawn. We used a sample of fawns studied by Sams et al. (1996b) as part of our data set to assess whether levels of TNF-α and IL-6 in blood serum of fawns would be useful predictors of risk of mortality.

Materials and Methods

Our study area was in Sequoyah State Park (35°30′N, 95°15′W) in eastern Oklahoma, where health and survival of neonatal fawns were monitored from 1990 to 1992. The primary vegetative association was deciduous forest of oak (*Quercus*)–hickory (*Carya*—Duck and Fletcher 1943; Sams 1994). Physical characteristics of the park (86% was bordered by a reservoir that limited emigration) and a no-hunting policy resulted in an overpopulated herd of white-tailed deer. Density estimates ranged from 39 deer/km^2 in 1991 to 80 deer/km^2 in 1989. Because of low levels of fecal nitrogen, suboptimal morphometrics, and physiologic parameters consistent with protein restriction, Sams (1994) concluded that deer at Sequoyah State Park suffered from chronic protein malnutrition.

Newborn fawns were captured by hand (McGuinnes and Downing 1969) and ages were estimated in days by measuring new hoof growth (Sams et al. 1996a). Fawns captured by 10 days of age were used in our analyses, unlike Sams et al. (1996b) who used only fawns captured by 3 days of age. We measured body mass, body length, and hind-foot length to determine intrauterine development. Whole blood was collected via jugular venipuncture into a 3-ml evacuated tube with potassium salt ethylenediaminetetraacetic acid (EDTA-K, Monoject, Sherwood Medical, St. Louis, Missouri), and serum was collected into a 10-ml evacuated serum-separating tube (SST, Becton Dickinson Vacutainer Systems, Rutherford, New Jersey). Serum was separated from blood by centrifugation ≤4 h after collection and stored for later analysis at −80°C. Hematologic analyses were performed ≤48 h after collection. We estimated tick loads by counting all ticks on ears of fawns and in a 2.5-cm diameter circular template that was placed over the anus and each eye.

Fawns were fitted with radiotransmitters (Advanced Telemetry Systems, Inc., Isanti, Minnesota) that were fastened to expandable collars. All fawns were visually relocated daily until 14 days of age and then remotely until 25 days of age. Cause of death (predation, emaciation, and so on) was determined when a mortality occurred by visual inspection of the area and postmortem examination (inspection of fat reserves, gastrointestinal contents, and hemorrhages or puncture wounds).

We manually counted total and differential leukocytes (Sams et al. 1993). Serum concentrations of gamma glutamyl transferase, gamma globulins, alkaline phosphatase, and hemolytic complement also were measured (Sams et al. 1996b). Means (± *SE*) differ from those reported by Sams et al. (1996b) because they used animals captured ≤3 days of age in their analyses, whereas we used animals captured ≤10 days of age to increase our sample size. We determined concentrations of serum TNF-α using a human-based, enzyme-linked immunosorbent assay as human-cytokine equivalents (Immunotech, Westbrook, Maine), and values were reported as immunoreactive TNF-α (iTNF-α). Bound enzymatic activity was measured at 414 nm using a plate reader (Titertek Multiskan II, Flow Laboratories Inc., McLean, Virginia). The interassay *CV* as described by the manufacturer (Immunotech) was 6.8% at 45 pg/ml. A similar enzyme-linked immunosorbent assay was used to determine human-cytokine equivalents of IL-6 (Cytoscreen US, Biosource International, Camarillo, California) in the serum, and values were reported as immunoreactive IL-6 (iIL-6). We measured bound enzymatic activity at 450 nm using a plate reader (Molecular Devices Corporation, Palo Alto, California). The interassay *CV* as described by the manufacturer (Biosour-

TABLE 1.—Risk factors associated with survival and mortality within 21 days of age for neonatal white-tailed deer at Sequoyah State Park, Oklahoma, 1990–1992.[a]

Parameter	Mortalities			Survivors		
	n	$\bar{X}$	*SE*	*n*	$\bar{X}$	*SE*
iTNF-α (pg/ml)[b]	13	688.4	168.8	32	412.9	81.2
iIL-6 (pg/ml)[c]	13	0.95	0.25	29	1.25	0.33
Body mass (kg)	13	2.94	0.35	32	3.63	0.18
Body length (mm)	13	621.5	21.3	32	681.0	10.2
Body mass/length^3 (g/dm^3)	13	11.8	0.9	32	11.4	0.4
White blood cell count (10^3/mm^3)	13	3.00	0.38	32	4.58	0.55
Lymphocytes (10^3/mm^3)	12	0.91	0.15	30	1.24	0.12
Neutrophils (10^3/mm^3)	12	2.15	0.35	30	3.30	0.50
Eosinophils (10^3/mm^3)	12	0.07	0.02	30	0.15	0.03
Monocytes (10^3/mm^3)	12	0.01	0.01	30	0.02	0.01
Hemolytic complement (CH_{50})	12	813	203	25	1,471	193
Gamma globulins (g/dl)	2	4.00	0.60	6	4.10	0.39
GGTP (IU/l)[d]	13	78.38	10.09	32	101.44	12.77
Alkaline phosphatase (IU/l)	13	1,066	144	32	1,244	105
Ticks (index)[e]	13	160.7	43.0	32	129.8	23.1

[a] Contains data from Sams et al. (1996b); means differ from those in Sams et al. (1996b) because different subsets of animals were used (see Materials and Methods).
[b] Immunoreactive tumor necrosis factor-α.
[c] Immunoreactive interleukin-6.
[d] Gamma glutamyl transferase.
[e] Tick index = ticks found attached to ears and within a 2.5-cm circular template placed over the eyes and anus.

ce) was 6.7% at 7.3 pg/ml. Human standards were used in assays because no standards exist for deer, and data were not corrected for differences in affinity to antibodies.

We compared serum concentrations of iTNF-α and iIL-6 between fawns that died at ≤21 days of age and those that survived >21 days using a Wilcoxon test. Pearson correlations were used to determine relationships between selected variables. We used logistic regression to determine if iIL-6 and iTNF-α were significant predictors of probability of mortality (P_m) to 21 days of age and developed a logistic regression model using stepwise forward selection of variables to predict mortality (Hosmer and Lemeshow 1989). Variables were permitted to enter and remain in the model in a hierarchical fashion when the $\log_e$ likelihood was deemed appropriate (α = 0.15—Bowyer et al. 1999; Sams et al. 1996b). Even more liberal levels of α (≥0.20) for selection of variables during model building in regression procedures have been suggested to increase the likelihood of selecting the best model (Bendel and Afifi 1977; Hosmer and Lemeshow 1989; Mickey and Greenland 1989). By setting too stringent an α-level during variable selection in stepwise logistic regression, some variables may not be considered appropriate for the model, although they may reliably predict the dependent variable when considered in concert with other variables (Hosmer and Lemeshow 1989:86). We tested suitability of our logistic regression model using a Hosmer–Lemeshow test for goodness-of-fit (Hosmer and Lemeshow 1989:140) where $P > 0.05$ indicated the model fit. All tests were performed using the Statistical Analysis System (SAS Institute Inc. 1990).

RESULTS

Mean levels of iTNF-α in sera of fawns that died before 21 days of age (688.4 pg/ml ± 168.8 *SE*, $n = 13$) tended to be greater than in survivors (412.9 ± 81.2 pg/ml, $n = 32$), although we did not detect a statistical difference ($P = 0.141$; Table 1). Levels of iIL-6 in serum did not differ ($P = 0.828$) between fawns that died before 21 days of age (0.95 ± 0.25 pg/ml, $n = 13$) and those that survived (1.25 ± 0.33 pg/ml, $n = 29$). Neutrophils were correlated positively ($r = 0.36$, *d.f.* = 53, $P = 0.007$) and lymphocytes were correlated negatively ($r =$

TABLE 2.—Logistic regression model[a] parameters for white-tailed deer dying before 21 days of age at Sequoyah State Park in eastern Oklahoma, 1990–1992.

Parameter	Estimate	*SE*	χ^2	*P*
Intercept	−4.380	3.642	1.446	0.229
Body mass/length^3 (g/dm^3)	−0.533	0.335	2.526	0.112
Hemolytic complement (CH_{50})	0.002	0.001	3.573	0.059
Gamma globulins (g/dl)	8.332	4.592	3.293	0.070
GGTP (IU/l)[b]	0.019	0.013	2.274	0.132
iTNF-α (pg/ml)[c]	−0.002	0.001	2.757	0.097

[a] The stepwise logistic regression model was significant ($P = 0.003$); Hosmer–Lemeshow test for goodness of fit indicated that the model fit ($P = 0.600$).
[b] Gamma glutamyl transferase.
[c] Immunoreactive tumor necrosis factor-α.

−0.34, *d.f.* = 53, $P = 0.011$) with concentrations of iTNF-α. We found that iIL-6 had a positive relationship with iTNF-α that was not quite significant ($r = 0.27$, *d.f.* = 51, $P = 0.056$). Parasite loads (ticks) were not correlated with either iTNF-α ($r = -0.17$, *d.f.* = 49, $P = 0.242$) or iIL-6 ($r = -0.10$, *d.f.* = 54, $P = 0.456$). We did not find relationships between P_m and serum concentrations of iTNF-α ($\chi^2 = 2.63$, *d.f.* = 1, $P = 0.105$) or iIL-6 ($\chi^2 = 0.35$, *d.f.* = 1, $P = 0.553$).

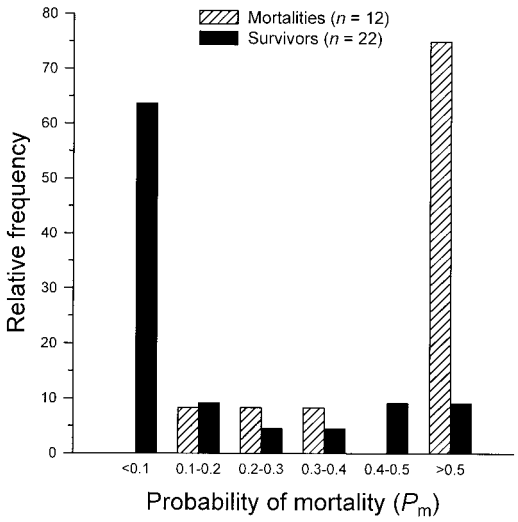

FIG. 1.—Predicted probabilities of mortality to 21 days of age for white-tailed deer fawns from Sequoyah State Park, Oklahoma, 1990–1992. Probabilities were calculated using a stepwise logistic regression model with weight/length^3, hemolytic complement, gamma glutamyl transferase, gamma globulins, tumor necrosis factor-α, and interleukin-6 as the predictive variables.

Stepwise logistic regression selected body mass/length^3, hemolytic complement, gamma globulins, gamma glutamyl transferase, and iTNF-α ($P = 0.003$) as the best predictors of mortality during the first 21 days of life (Table 2). The original model included iIL-6 as well, but when recalculating statistics after completion of the model-building process, iIL-6 was not significant ($P = 0.161$) and was dropped from the model. Based on the logistic predictive equation, fawns dying before 21 days of age had a P_m of 0.163–0.998 (0.604 ± 0.074, $n = 12$), and those surviving had a P_m of 0.001–0.916 (0.163 ± 0.053, $n = 22$; Fig. 1). The Hosmer–Lemeshow test for goodness-of-fit indicated that the model fit the data well ($P = 0.60$). The stepwise logistic model correctly predicted 75.0% of the mortalities and 81.8% of the survivors at $P_m > 0.4$ (Table 3). Of the 3 mortalities that were predicted to be survivors, 2 died from emaciation and 1 died from unknown causes. Increasing P_m to 0.5 increased sensitivity of the model, resulting in 90.9% of survivors being predicted accurately.

DISCUSSION

Analysis of our data suggests that neither TNF-α or iIL-6 alone are useful predictors of elevated probability of mortality due to physiologic stress in neonatal white-tailed deer from high-density populations. This

TABLE 3.—Predictive power of a logistic regression model[a] designed to predict probability of mortality (P_m) at $P_m \geq 0.4$ and $P_m \geq 0.5$ in neonatal white-tailed deer to 21 days of age at Sequoyah State Park, Oklahoma, 1990–1992. The model was developed using values for mass/length^3, hemolytic complement, gamma glutamyl transferase (GGTP), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and gamma globulin concentrations.

	$P_m \geq 0.4$ predicts mortality		$P_m \geq 0.5$ predicts mortality	
	Mortalities	Survivors	Mortalities	Survivors
Actual observation	12	22	12	22
Predicted				
Mortalities	9	4	9	2
Survivors	3	18	3	20
Mortalities predicted accurately (%)[b]	75.0		75.0	
Survivors predicted accurately (%)[c]	81.8		90.9	
Predicted as mortalities that died (%)[d]	69.2		81.8	
Predicted as survivors that survived (%)[e]	85.7		87.0	

[a] $P_m = e^a/1 + e^a$, where a = [4.380 + 0.533(weight/length^3) − 0.002(complement) − 8.332(gamma globulins) − 0.019(GGTP) + 0.002(TNF-α)].

[b] Proportion of actual mortalities that the logistic regression model predicted as mortalities.

[c] Proportion of actual survivors that the logistic regression model predicted as survivors.

[d] Proportion of individuals predicted by the logistic regression model to be mortalities that were actual mortalities.

[e] Proportion of individuals predicted by the logistic regression model to be survivors that were actual survivors.

partially may be a factor of the limited statistical power of this study and the inherent variability associated with investigations of free-ranging animals. Nevertheless, high levels of iTNF-α in fawns that died before 21 days of age suggested that they were subjected to elevated levels of parasitic infection, but we did not conduct a complete screen for internal parasites. Pathologic examinations did not reveal severe pathogenic challenges in fawns that died, and iTNF-α was not correlated with tick burdens. Sams et al. (1996b) noted that fawns in this population that possessed an increased risk of mortality before 21 days of age had poorly developed immune systems. Those individuals had low levels of gamma glutamyl transferase and gamma globulins, suggesting at least a partial failure in passive transfer of immunity from the mother during the first 24 h of life. Assuming that a fawn with a poorly developed immune system would be more susceptible to pathogens seems reasonable, and we would expect elevated levels of iTNF-α in challenged individuals because of inflammatory response.

Of the individuals that died before 21 days of age, 7 (58.3%) died from emaciation, a common characteristic of individuals with elevated levels of iTNF-α. TNF-α, also known as cachectin, has been shown to cause body wasting, or cachexia, during chronic disease when circulating levels become extremely elevated (Beutler et al. 1985; Tracey et al. 1988). During inflammatory processes such as those commonly associated with parasitic infection, TNF-α can stimulate mobilization of peripheral energy reserves in support of metabolic demands associated with an inflammatory response. However, extended periods with elevated levels of TNF-α contribute to cachexia, which is symptomatic of many chronic disease states (Sherry and Cerami 1988).

Contrary to our hypothesis, iIL-6 was a poor predictor of mortality during the first 21 days of life. Protein nutritional stress, as indicated by low mass at birth (Sams 1994), possibly compromised mechanisms of IL-6 production and release in neonates, despite infection. Spaulding et al. (1997) noted that caloric restriction depressed circulating concentrations of IL-6 in laboratory mice.

They found that laboratory mice that were 50% calorie-restricted had concentrations of IL-6 about 75% less than control mice. Sams et al. (1996b) noted that fawns that did not survive >21 days likely had received poor passive transfer of immunity during the first 24 h of life and attributed this to malnutrition of adult females during late gestation. Given energetic costs of lactation (Robbins 1993), females may have had reduced milk production resulting from nutritional restriction (Allden 1970; Thomson and Thomson 1953), subsequently causing reduced intake of milk and growth of fawns (Kitts et al. 1956; Murphy and Coates 1966; Verme 1963). Sams et al. (1996b) also noted that those fawns that did not receive sufficient colostrum also had depressed development in utero. Fawns with low birth mass commonly have difficulty nursing (Verme 1962), further increasing their level of nutritional restriction.

Age also can have a pronounced effect on circulating concentrations of cytokines. Most cytokines are regulated closely in young animals and typically are present at very low levels in sera of newborns (Spaulding et al. 1997). Effros et al. (1991) noted that production of IL-6 by 6-month-old laboratory mice was about 33% that of mice ranging in age from 9 to 12 months. Our fawns were <10 days of age when captured, which may explain why many had concentrations of iIL-6 below detection levels.

Although iTNF-α alone was not statistically correlated with mortality, in combination with other variables (body mass/length^3, hemolytic complement, gamma globulins, gamma glutamyl transferase), it predicted probability of mortality. These results highlight the potential value of TNF-α as a tool to assess stress (i.e., nutritional, immunologic). Further research should focus on developing a complete understanding of circulating levels of cytokines in neonates and responses of those cytokines to stresses frequently encountered by free-ranging neonates. Additional effort to elucidate relationships between immunologic parameters and neonatal mortality may clarify our understanding of density dependence and mortality in high-density populations.

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