

Acute Phase Proteins and Cytokines in Alaskan Mammals as Markers of Chronic Exposure to Environmental Pollutants

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Abstract.—We examine the usefulness of developing acute phase proteins and cytokines as markers of biological effects for free-ranging Alaskan mammals. Blood assays for these markers are already in widespread use as indicators of chemical pollution exposure, disease, and stress in humans. The acute phase response includes fever and changes in the plasma concentration of several proteins that originate in the liver. The acute phase response is mediated by cytokines, such as interleukin-1 and interleukin-6, which are released by macrophages and other cells. The few data available indicate that these biochemical markers are valuable for documenting chemical exposure when used in conjunction with an overall biostatistical model. Additional baseline studies using this nonlethal, analytical method for mammal populations are needed to characterize age, sex, and season as sources of variation.

After stress or exposure to pollutants such as crude oil, animals may exhibit an immediate or acute response and a chronic response. Both of these responses affect various homeostatic mechanisms in vertebrates, the most significant being that on the immune system. Any secondary infection or tissue damage will lower the long-term survivorship of the organism and, if widespread, hold consequences for the population. Activity of the immune system can be monitored by measuring various blood components, including plasma proteins, enzymes, and cytokines. To develop mammalian plasma and cytokines as markers of biological effects, extensive laboratory and controlled field studies are needed to demonstrate that these markers show a reproducible response to oil exposure. Also, the variation in response attributable to differences in age, sex, and nutritional state must be characterized. Because these types of studies have only begun for Alaskan mammals, the major focus of this article is to examine changes observed in the pattern of acute phase proteins and cytokines after exposure to environmental hydrocarbons, as occurred after the *Exxon Valdez* oil spill in Prince William Sound.

The Acute Phase Response

The acute phase response (APR) is nonspecific and commences within hours of inflammatory stimuli and results in the increased synthesis of certain plasma proteins (Kushner 1982). During APR, a negative nitrogen balance and decreased synthesis of anabolic lipogenic enzymes occurs in association with fever (Atkins 1984; Lowry et al. 1992). Likewise, there is a change in the viscosity of blood as

determined by the sedimentation rate of red blood cells (Bull 1981). This change in sedimentation rate has been well documented in man and more recently in sea otters *Enhydra lutris* exposed to crude oil spilled from the *Exxon Valdez*; Wilson et al. (1990) reported that this rate increased almost four fold in sea otters and observed that anemia and hemolysis, which could trigger APR, lasted for 90 d in oil-exposed sea otters.

Acute phase proteins are defined by the magnitude of change in their concentration after inflammation or infection (Atkins 1984; Kushner and MacKiewicz 1987). The change in concentration of some APR proteins can occur as early as 6 h and last up to 2 weeks under controlled laboratory conditions. Most APR proteins are glycoproteins, with the notable exceptions of albumin, C-reactive protein, and serum amyloid A protein (SAA). Although most APR proteins have been documented only in humans and laboratory animals (such as rats) SAA has been extensively studied in the mink *Mustela vison* (Marhaug et al. 1984), and in horses *Equus caballus* (Husebekk et al. 1986; Pepys et al. 1989). In the circumpolar north, the mink is a likely candidate for the monitoring of environmental changes caused by pollution (Edqvist et al. 1992). The ease of capture and recapture, widespread distribution, high reproductive rate, and abundance makes them effective biological monitors for long-term effects that may occur over several years and generations (Talmage and Walton 1991).

The increase of APR proteins in plasma after inflammatory stimulus results from increased rates of synthesis and secretion by liver parenchymal cells

TABLE 1.—Selected blood components for river otters inhabiting oiled ($N = 11$) and non-oiled ($N = 11$) areas of Prince William Sound in 1991. Part of this data has been published previously (Duffy et al. 1994). We examined relationships among blood values with simple correlations (Dixon 1985). These and other blood values were used to classify otters from oiled and non-oiled areas using logistic regression (Agresti 1990). Abbreviations are Hb = hemoglobin and IU = international unit.

Blood components	Oiled		Non-oiled	
	Mean	SE	Mean	SE
Interleukin (IL-6ir, pg/ml)	48.3	13.8	17.3	11.3
Interleukin (IL-1ir, pg/ml)	13.3	6.6	10.1	6.1
Haptoglobin (Hp, Hb binding/dL)	156.9	27.9	30.0	15.6
Alanine aminotransferase (ALT, IU)	152.7	8.8	138.5	14.6
Aspartate aminotransferase (AST, IU)	437.2	70.0	418.1	67.0
Lactate dehydrogenase (LDH, IU)	146.2	25.2	154.0	43.1
Creatine kinase (CK, IU)	3,038.6	820.8	1,885.8	516.4
Hemoglobin (Hb, g/dL)	16.3	0.6	15.7	0.6
Packed cell volume (PCV, ml/mm ³)	42.9	1.6	44.1	1.6
Sodium (Na, meq/L)	148.5	3.0	156.6	4.7
Potassium (K, meq/L)	4.1	0.1	4.5	0.1
Chloride (Cl, meq/L)	117.1	2.6	123.5	4.6
Calcium (Ca, mg/dL)	9.2	0.2	9.8	0.6
Phosphate (PO ₄ , mg/dL)	5.5	0.3	5.7	0.7
Blood urea nitrogen (BUN, mg/dL)	36.1	3.4	36.7	4.9
Creatine (CREA, mg/dL)	0.45	0.03	0.60	0.46
Cholesterol (Chol, mg/dL)	161.7	9.4	221.5	26.1
Bilirubin total (mg/dL)	0.27	0.05	0.32	0.07
Direct bilirubin (mg/dL)	0.16	0.05	0.20	0.08
Total protein (Prot., g/dL)	7.4	0.4	7.6	0.3
Albumin (Alb, g/dL)	3.1	0.2	3.1	0.1
Glutamyl transferase (GGT, IU)	20.7	2.8	20.1	2.7
Alkaline phosphatase (AP, IU)	226.2	25.3	262.9	55.3

(Pepys and Baltz 1983). Perlmutter et al. (1985) have shown that cells other than hepatocytes also synthesize these proteins, but that extrahepatic synthesis plays only a minor role in elevating blood levels of these proteins. Acute phase response proteins made in nonhepatocytes probably act locally in a particular tissue such as muscle or brain (Dinarello 1984; Tamm 1989).

Wide normal ranges and variability in the response of individual APR proteins has been observed between different species of animals (Sheldrick et al. 1982; Conner et al. 1986; Schreiber et al. 1989). In most animals studied, however, there always appears to be an enhanced synthesis of haptoglobin (Hp), alpha-1-acid glycoprotein (AG), and fibrinogen (Fb) and reduced albumin synthesis (Conner et al. 1986; Kushner and MacKiewicz 1987; Allen and Kold 1988; Schreiber et al. 1989). Other protein levels seem to vary between species; for example, fibronectin is an APR protein in rodents but not in man (Dyck and Rogers 1985). Approximately two-fold variability in response of individual APR proteins also has been related to the sex of the animal (Bosanquet et al. 1976), which may be in response to synergistic action of hormones and cytokines. There is a general lack of data on APR proteins and the level of concentration changes in

marine species and aquatic wildlife such as mink, river otters *Lutra canadensis*, seals, and whales, which are very sensitive to environmental pollution (McIntyre and Pearce 1980; Dieter and DiGiulio 1989; Duffy et al. 1993). Limited comparative studies suggest that the physical structure of APR proteins may have been preserved through millions of years of evolution (Ledoux and Kenyon 1975; Kurosky et al. 1976; Atkins 1984; Wahl et al. 1992) and presumably is vital to survivorship of the organism.

In an *Exxon Valdez* study, river otters from oiled areas exhibited significantly higher levels of Hp, indicative of physiological damage, and were significantly lower in body mass than otters from non-oiled areas of Prince William Sound. River otters might be exposed to oil by grooming it from their fur, eating oil-covered prey, or ingesting food (e.g., mussels *Mytilus* sp.) that had concentrated hydrocarbons (Bowyer et al. 1994; Moore 1985). Elevated blood values (Table 1) and reduced body mass for otters from oiled sites might result from internal damage from oil, reduced digestibility of oiled food, a reduction in prey, or some combination of these (Duffy et al. 1993, 1994a). Haptoglobin values differed in animals from oiled and non-oiled regions of Prince William Sound for 2 years but may have recovered in the third year (Duffy et al. 1994b).

Haptoglobin values for river otters obtained in 1991 and 1992 are substantially lower than those from 1990. This may have occurred for two reasons: first, the Hp response elicited by exposure to crude oil may be waning over time; or second, samples were collected in late winter in 1990 but during summer in 1991 and 1992. Although the effects of exposure to oil probably would diminish over time, this does not explain why Hp levels declined markedly from 1990 levels in animals from both oiled and non-oiled areas. Thus, the most likely answer is that Hp levels are the result of complex interactions with other environmental factors that vary seasonally.

Cytokines and the Mediation of Acute Phase Response Protein Synthesis

Absorption and exposure of tissue to environmental pollutants such as crude oil can lead to local tissue injury. In Prince William Sound, tissue injury was observed in sea otters, as indicated by cell analysis and leakage of enzymes such as creatine kinase (Williams et al. 1990). Also, 1 year after the spill, increased level of liver enzymes, such as AST, leaking into the plasma was observed in river otters (Table 1; Duffy et al. 1994a). Injury of tissue is known to cause APR and is mediated by messengers called cytokines (Lowry et al. 1992).

Cytokines are small protein molecules (molecular weight <20 kilodaltons) that are synthesized and secreted by different cells, most notably those of the immune system such as macrophages. Interleukins are cytokines used by immune and inflammatory cells to communicate with each other and are produced at the site of infection or injury. They act locally on other cells (paracrine) or may circulate, causing systemic effects. There are now more than eight different cytokines with their receptor systems reported (Arai et al. 1990). Several of these cytokines induce APR, and it is becoming clear that the synthesis of individual APR proteins is a complex process of control. These molecules exert a wide range of biological effects on cells, allowing cells of immune, hemopoietic, and neuronal systems to communicate (Dinarello 1984; Machiewicz et al. 1991; Kishimoto et al. 1992). Not only do cytokines function pleiotropically, but they also operate in a redundant manner through a tyrosine kinase-linked receptor system (Mantovani et al. 1992; Taga and Kishimoto 1992).

Early interest focused on the role of interleukin-1 (IL-1) in mediating APR. Interleukin-1 leads to the production of IL-6, which has been shown to induce the synthesis of Hp. Detectable levels of IL-1-im-

munoreactive molecules (IL-1 α) have been observed in seals from the Prince William Sound area, which may have been exposed to crude oil (L. K. Duffy, Institute of Arctic Biology, and K. J. Frost, Alaska Department of Fish and Game, unpublished). Increased IL-1 levels may lead to local APR response in the brain and demyelination by macrophages (Said and Hontebeyrie-Jaskowicz 1992). Furthermore, IL-1 α was detected in river otters but did not differ significantly between river otters living in oiled and non-oiled areas (Table 1; Duffy et al. 1994a). An IL-1-like substance also has been reported in fish and lizards (Kluger et al. 1975; Bernherm et al. 1978; S. Trask and Duffy, unpublished).

Our knowledge of the role of IL-6 in inflammation, viral infection, autoimmunity and cancer continues to increase. Abnormal IL-6 production has been associated with autoantibodies in human patients with benign heart tumors, and increased IL-6 production has been documented in rheumatoid arthritis. Viral infections such as that caused by the human immunodeficiency virus (HIV) lead to high serum concentrations of IL-6 (Breen et al. 1990). In wildlife populations, differences in IL-6 α have been observed between river otters living in oiled and those living in non-oiled areas of Prince William Sound in 1991 (Table 1; Duffy et al. 1994a). The importance of monitoring the immune system after chemical exposures has been repeatedly documented in humans and should be expanded to free-ranging mammals (Weeks et al. 1992).

Monitoring Acute Phase Response Proteins and Cytokines

To predict the environmental risk, the probability of deleterious effects must be estimated. Because the APR largely reflects plasma concentrations of Hp, fibrinogen, and other acute phase proteins, the direct measurement of these proteins to monitor chronic inflammation would be a useful diagnostic aid in following the course of exposure to pollutants and can be used to provide evidence of exposure of mammals to such pollutants (Duffy et al. 1994a). With respect to Prince William Sound, stepwise logistic regression using Hp, IL-6 α and a liver enzyme correctly classified 86.4% of 22 river otters as inhabiting oiled or non-oiled areas. Moreover, otters living in oiled areas abandoned use of existing latrine sites at a rate that was more than three times greater than in non-oiled areas, strongly suggesting that the predictive model based on blood values reflected changes in this otter population (Duffy et al. 1994a). More studies employing probability the-

ory and different acute phase proteins will be required to identify how many acute phase proteins should be monitored. Haptoglobin and other acute phase proteins such as alpha-1-antichymotrysin inhibitor, whose function can be measured quantitatively, are useful in wildlife species where immunochemical probes are not yet available or validated. A probe for C-reactive protein levels, diagnostic of human patients with chronic inflammatory states and leukemia, would be especially useful in studying sea otters, river otters, or other large mammals exposed to environmental pollutants. Monitoring APR proteins would aid workers in oil spill recovery facilities when following the health of oiled animals before they are released into the wild. If long-term survivability is to be studied, measurement of cytokines such as IL-6 and tumor necrosis factor (TNF) would be useful to show abnormal, chronic stimulation of the immune system (Dinarello 1984; Breen et al. 1990; Lowry et al. 1992).

Understanding the responses of river otters to oil contamination obviously requires pre-spill data and long-term monitoring. Duffy et al. (1994a, 1994b) detected changes in blood values of river otters that are related to their health more than 2 years after the spill. This raises important questions about the long-term effects of oil on river otters and other mammals similarly exposed to crude oil. The validity of the approach is indicated by year-3 data, which show potential recovery in the oiled population of river otters. Because of their high position in the food chain, river otters and mink can serve as "indicator species" for monitoring the health and recovery of animals at lower trophic levels in Prince William Sound.

In the years immediately after the *Exxon Valdez* oil spill much attention has been given to the problem of restoring the damaged ecosystem in Prince William Sound. Nonlethal monitoring techniques using blood samples and recapture methods allow fish and wildlife managers to observe the health of the animals without removing them from the population (Fossi and Leonzio 1994). In the context of a nonlethal monitor of the environment, acute phase proteins and cytokines may be found to serve as an early warning signal of possibly more serious pathologies, especially if reexposure to chemical pollutants and environmental pathogens is prolonged.

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