Environmental pollutants and marine mammal health: The potential impact of hydrocarbons and halogenated hydrocarbons on immune system dysfunction

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ABSTRACT

This paper provides a detailed review of the immunotoxicological effects of environmental pollutants on the health of marine mammals, particularly in relation to their impact on the immune system and mechanisms of toxicity. Environmental pollutants are increasingly implicated (both directly and indirectly) with the onset of infectious disease and related mortality incidents in marine mammals,. The release of chemicals into the marine environment and the subsequent bioaccumulation up the food chain may pose a serious threat to marine mammals inhabiting contaminated areas; this has been documented in various studies of pollutant concentrations in tissue samples and large scale mass mortalities. Data correlating pollutant residues with altered reproductive/developmental states, and immune system dysfunction in particular, are reported for terrestrial mammals and suggest a similar association in marine mammals. Immunology is emphasised as a tool for assessing marine mammal health using quantitative and qualitative techniques to establish the effects of chemical pollutants. This has become increasingly important in relation to the subsequent dangers that may be posed to humans through any indirect exposure via the food chain.

KEYWORDS: POLLUTANTS; ORGANOCHLORINES; DISEASE; REVIEW; IMMUNOSUPPRESSION

INTRODUCTORY CONCEPTS OF IMMUNOTOXICITY IN MARINE MAMMALS

During the last twenty years there has been increasing attention directed toward the role of chemical pollutants as causative factors in the onset of disease. This has been generated, at least in part, by intense media coverage of accidental and deliberate chemical releases into the environment, and has been focused primarily on the initiation of disease in humans exposed to environmental pollutants and in seals and sea lions dying from viral diseases. Critical incidents have led to the association between chemical exposure and diseases in humans. However, this attitude is less prevalent with respect to consideration of the widespread pollution of the oceans, or the bioaccumulation of lipophilic chemicals up the food chain resulting in detectable levels of chemical pollutants in marine animals. However, with the increased availability of information on the effects of eating pollutant-contaminated

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seafood, it is becoming generally recognised that marine animals may also suffer both directly and indirectly from diseases caused by chemical pollutants, for example as a result of the loss of immune system protection against micro-organisms in the environment. Few direct data exist which correlate pollutant residues in marine mammal tissues with altered reproductive or developmental states, or with depressed immune function and increased levels of disease (Reijnders *et al.*, 1999). However, a number of physical entities and chemical agents are known to initiate immunosuppression in other animals, including radiation, chemotherapeutic agents, immunointeractive viruses and some of the chemicals found as environmental pollutants. Many chemicals have been clearly established to have immunotoxic properties in some laboratory animals. The following sections will emphasise those chemicals known to be immunosuppressive in terrestrial mammals, including man. Their distribution is reviewed from the perspective of what types of chemicals exist in the environment, whether they are found in marine mammals and how they might interact either directly with cells of the immune system or with physiological mechanisms regulating the immune system. This review also summarises some of the basic concepts in immunology and discusses the types of immunologically-based investigative tools and data that will be needed to accurately determine the effects of chemical pollutants on immune function in marine mammals.

ENVIRONMENTAL POLLUTANTS: IMPACT ON MAMMALS

Background

The hydrocarbons, including aromatic hydrocarbons, polycyclic aromatic hydrocarbons and halogenated hydrocarbons, include several broad groups of chemicals extensively used in agriculture and industry and widely disseminated as pollutants (Kimbrough and Jensen, 1989). A large number of these compounds, including members of the polychlorinated biphenyl (PCB), polybrominated biphenyl (PBB), naphthalene (PCN), benzene, phenol, and terphenyl (PCT), polynuclear aromatic hydrocarbon (PAH), halogenated phenols, anilines, benzenes, dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and chlorinated pesticides, insecticides and herbicides have been found as pollutants at terrestrial and aquatic sites. The stability and lipophilicity of such chemicals result in their tendency to be concentrated in fatty tissues, leading to their bioaccumulation up the food chain. Detectable residue levels of a variety of organochlorines and aromatic hydrocarbons, including the PCBs, have been identified in the liver, milk and adipose tissues of both terrestrial and aquatic animals, and in human adipose, milk and serum (Safe *et al.*, 1985).

The PCBs comprise a large group of 209 isomers and congeners which have two to ten chlorine atoms substituted in the phenol rings and differ only in the number of chlorine residues and their positions on the biphenyl structure (Mullin *et al.*, 1984). PCBs were originally synthesised in 1881 for commercial use where a stable oil with a high flash point was required. They were used for cutting oils and heat transfer oils, in generators and transformers and in a variety of industrial formulations and processing procedures. Their use was restricted in the USA in the early 1970s and banned throughout much of the industrial world by the late 1970s. However, considerable quantities of the total global PCB production is still in use in closed systems (Van der Gaag and Marquenie, 1991). Although the accuracy of the proposal has been questioned (S. Safe, pers. comm.), Peterle (1991) suggested that a number of industrialised countries might still have synthesised PCBs into the 1990s. Public awareness of and concern for the ecosystem damage and potentially adverse health effects resulting from exposure to the variety of PCBs and related organochlorine pollutants was minimal until the last two decades. That awareness has increased significantly with reports of human agricultural and occupational exposures (e.g. Cook *et al.*, 1980; Brown and Jones,

1981; Hardell, 1981; Suskind, 1983; 1985; Gustavsson *et al.*, 1986), a series of PCB poisonings in Asia resulting in immunosuppression (Kashimoto *et al.*, 1981; Chen and Hsu, 1987), the broad media discussion of the dioxin contaminant controversy (Patterson and Hoffman, 1976; Kimbrough and Carter, 1977), the widely reported health problems in Vietnamese populations and American military personnel resulting from Agent Orange contamination (Lawrence *et al.*, 1985; Lathrop *et al.*, 1987) and the toxic effects of a PBB contamination in Michigan (Reich, 1983).

The PCBs were initially identified by Jensen (1966) as a tissue residue in seals three decades ago, with significant bioaccumulation occurring from ingestion of PCB-contaminated fish. The ocean serves as a major reservoir for pollutant PCBs. While the time of residence in any one oceanic compartment may be relatively short, the persistence of PCBs allows for recycling between different geographical areas and system components before being removed through metabolism or photodecomposition (Manchester-Neesvig and Andren, 1989). It has been estimated that 61% of the total environmental load of PCBs is found in the marine ecosystem, but the overall marine concentration of PCBs tends to be low, on the order of parts per trillion. These calculations are complicated by the fact that up to 80% of pollutant PCBs in the total marine environment are concentrated in the North Atlantic (Tanabe, 1988). Virtually all of the PCBs are considered to be toxic to one degree or another, but only 20 of the 209 known isomers and congeners found in PCB mixtures have planar, non-*ortho*, chlorine substitutions in the biphenyl ring. Of these, only three, 3,3',4,4'-tetrachlorobiphenyl, 3,3',4,4',5-pentachlorobiphenyl and 3,3',4,4',5,5'-hexachlorobiphenyl (IUPAC No. 77, 126 and 169 respectively; Fig. 1) are proximate isostereomers of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The planar non-*ortho* PCBs are thought to be responsible for most of the toxic effects of PCBs on biological organisms (Hansen, 1987; Safe, 1990; McKinney *et al.*, 1992), with the determination of toxicity between PCBs being, at least in part, associated with the fact that non-*ortho* congeners interact with the *Ah* receptor, while congeners with *ortho*-chloro substituents are inactive as *Ah* receptor agonists.

While animals taken directly from contaminated sites may have high tissue concentrations of lipophilic chemicals, as evidenced by PCB concentrations as high as 600ppm in the fat of white whales in the St. Lawrence Estuary (e.g. Massé *et al.*, 1986), marine mammals sampled in virtually all oceans, including some pristine waters generally considered to be uncontaminated, have also been found to have high, although not acutely toxic, organochlorine residues in their tissues (Aguilar *et al.*, 1999). The physiological consequences of chronic cellular exposure to potentially toxic organochlorines and hydrocarbons are unknown; however, elevated PCB tissue residues are reported to have occurred concurrently with the recent mass mortalities of dolphins in the United States (Geraci, 1989; Kuehl *et al.*, 1991; 1994) and with the 1990 western Mediterranean epizootic dolphin deaths, where PCB levels as high as 3,000ppm were detected (Aguilar and Borrell, 1994). An initial report suggests the possible existence of a cause-effect relationship between tissue residues of organochlorines and decreased mitogen-initiated lymphocyte blastogenesis in cetaceans (Lahvis *et al.*, 1993). The relationship certainly has been shown in humans (Chen *et al.*, 1985; Chen and Hsu, 1987; Kashimoto and Miyata, 1987), but cannot be assumed for marine mammals from the very limited experimental data. Considerable data/sets from most ocean areas reveal residues of persistent organochlorines in tissues of marine mammals, but with few exceptions, studies do not address the physiological consequences of chemical residues, and neither support nor refute the proposed association between chemical exposure and immune system dysfunction. Data do, however, show that organochlorine and hydrocarbon pollutants act as inducers of cytochrome P450s in marine vertebrates, including dolphins (Geraci and St. Aubin, 1982; Geraci, 1990), that cytochrome
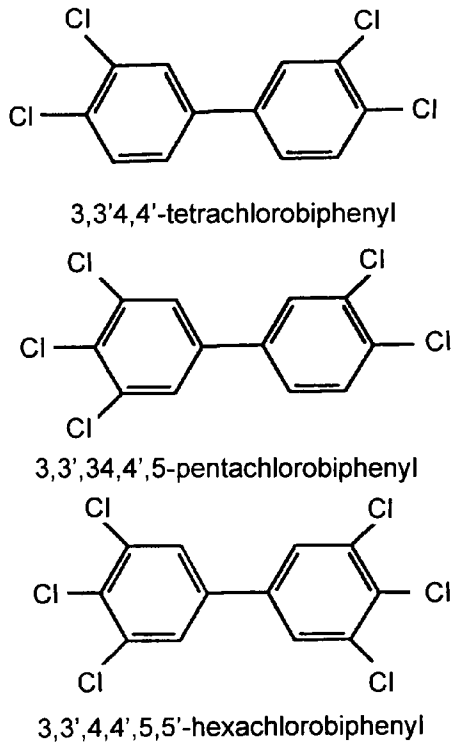
3,3'4,4'-tetrachlorobiphenyl

3,3',34,4',5-pentachlorobiphenyl

3,3',4,4',5,5'-hexachlorobiphenyl

Fig. 1. Three polychlorinated biphenyls, 3,3',4,4'-tetrochlorobiphenyl, 3,3',4,4',5-pentachorobiphenyl, and 3,3',4,4',5,5'-hexachorobiphenyl, IUPAC No. 77, 126 and 169 respectively, which are proximate isostereomers of 2,3,7,8-tetrachorodibenzo-*p*-dioxin.

P450s metabolise aromatic hydrocarbons to form electrophilic metabolites capable of causing DNA adducts (Poland *et al.*, 1979; Poland and Knutson, 1982; Safe, 1984; 1990; Whitlock, 1986; 1987) and that DNA-hydrocarbon adducts have been reported for cetaceans from both pristine and polluted waters (e.g. Ray *et al.*, 1991). These data tend to support the proposal by Payne *et al.* (1987) that hydrocarbon induction of cytochrome P450 enzyme systems may serve as an early warning indicator qualifying as a 'most sensitive biological response' for evaluating the presence of organic pollutants in marine ecosystems.

This brief review does not address the impact of oil and oil spills on marine mammals. Geraci (1990) compiled an extensive review of the effects of oil on marine mammals. The studies considered the potential for oil as an agent leading to increased mortality in marine mammals due to physical and physiological alterations, but did not address the potential effects of oil and oil-derived chemicals on immune system function or the overall health of the animals.

Environmental impact of hydrocarbon pollutants

Most organochlorine pollutants exhibit similar chemical and environmental properties. They are stable lipophilic compounds that are only slowly degraded by acids, bases, heat and oxidative processes, resulting in their being persistent and in their tendency to bioaccumulate up the food chain. For organochlorines, the degree and position of halogen substitution is directly related to stability and lipophilicity, as well as to toxicity (Goldstein and Safe, 1989)

and immunotoxicity (Saboori and Newcombe, 1992). Mechanisms by which the organochlorines initiate toxic phenomena are diverse; however, for a number of organochlorines, interactions with the *Ah* receptor are required to elicit toxic phenomena (see below). Specific classes of polycyclic aromatic hydrocarbon and halogenated hydrocarbon pollutants, including the PCBs (with 209 possible congeners), PCDFs (with 135 possible congeners), PCDDs (with 75 possible congeners) and a wide variety of PAH, have been identified as significant environmental contaminants. Of these, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; Fig. 2) is the most toxic. TCDD has a reported ED_{50} (the concentration at which a 50% determination of response occurs) that may be as low as 10^{-13} M dependent on the test species, and has been described as a cytochrome P450 inducing agent and a suspected carcinogen (Schwetz *et al.*, 1973; Whitlock, 1987; Safe, 1990). Different species vary greatly in their responses to TCDD, with guinea pigs showing
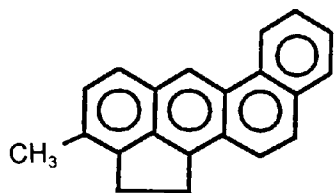
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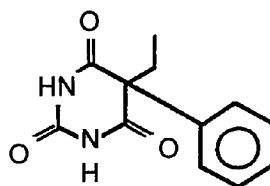
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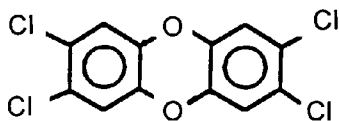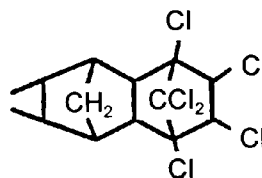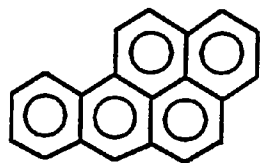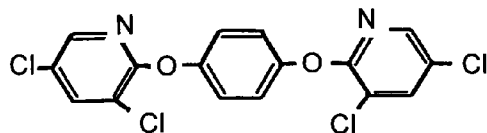
2,3,7,8-tetrachlorodibenzo-p-dioxin
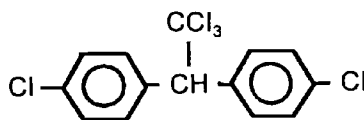
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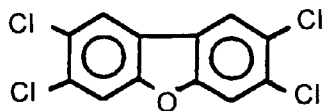
2,3,7,8-tetrachlorodibenzofuran

dieldrin

benzo(a)pyrene

1,4-bis[2(3,5-dichloropyridyloxy)]benzene

Fig. 2. A partial list of polycyclic aromatic hydrocarbons (PAH) and halogenated hydrocarbons (organochlorines) that are carcinogens, or are cytochrome P450 inducing chemicals, or interact with basic helix-loop-helix interactive cellular receptors.

an LD_{50} two orders of magnitude lower than some mouse strains (Esposito *et al.*, 1980). These differences have contributed to significant disagreement about the actual concentration of TCDD required to produce toxic effects. Nevertheless, it is clear that TCDD (and related compounds) is toxic, ubiquitous, and, with a reported biological half-life in humans of approximately ten years (Poellinger, 1995), persistent. Environmentally dispersed organochlorines such as TCDD and related compounds are clearly of concern and represent a long-term threat to aquatic animals in waters bordering both industrialised countries and densely populated emerging countries.

When animals come in contact with TCDD, the sequence of physiological effects can be complex and differ dramatically between species. In 1971, TCDD contaminated oil was used to spray multiple sites in Missouri for dust control, including a show ring where a number of animal deaths subsequently occurred. Autopsies of seven horses which died within weeks after the application of oil to the ring revealed oral ulcers, gastric ulcers, ascites, hyperkeratosis, nephritis and cystitis, with the onset of illness days to weeks after initial exposure, and deaths progressing over a period of several weeks (Kimbrough and Carter, 1977). Numerous other animals, including dogs, cats, mice and birds died in or around the ring within that time period, and a number of humans became ill with persistent headaches and skin lesions. Analyses of soil collected from the show ring revealed TCDD concentrations of 30ppm, with TCDD concentrations at 1ppb in up to 42 other affected sites. An extensive study did not indicate the existence of depressed B or T cell blastogenesis in TCDD-exposed human populations (e.g. Patterson and Hoffman, 1976); however, thymosin alpha-1 levels completed on serum frozen for three years suggested that the TCDD exposed group, which were depressed from 1,148.7±482 pg/ml in unexposed controls to a low of 9.73±304 pg/ml in the exposed group, apparently demonstrated decreased thymic function associated with TCDD exposure (Stehr-Green *et al.*, 1989; Hoffman, 1992).

Such clinical data are consistent with experimental findings (Morris *et al.*, 1993; Wood *et al.*, 1993) showing that TCDD significantly suppresses IgM secretion and background proliferation in both human and marine animal B-cells. The proposal that TCDD in some way interferes with cell maturation is further supported by data showing that the *Ah* receptor-dependent depression of immune function may be due to alteration in the maturation rate of thymocytes associated with organochlorine-initiated changes in thymocyte interactions with epithelial cells (Esser and Welzel, 1993). Investigations of marine thymocyte maturation further suggest that 3,3',4,4'-tetrachlorobiphenyl (one of the non-*ortho* isostereomers of TCDD) inhibits proliferation of thymocytes, perhaps by enhancing premature differentiation of thymocytes into cytotoxic T lymphocytes (Lai *et al.*, 1994). TCDD treatment and determinations of function in T-cells from *Ah* receptor positive and *Ah* receptor negative mice suggested that inhibition of splenic plaque-forming-cell responses was primarily *Ah* receptor-independent, while antigen-mediated immune responses were apparently sensitive to *Ah* receptor-dependent immunotoxicity of halogenated hydrocarbons (Harper *et al.*, 1993). Thus, the organochlorines appear to have at least two generalised groups of mechanisms for interfering with physiological functions including function of the immune system. One family of mechanisms is apparently dependent on xenobiotic binding to the *Ah* receptor. At least one more family is independent of *Ah* receptor binding, but is associated with binding to one or more other classes of cellular receptors.

Xenobiotics that do not bind (or bind with low affinity) to the *Ah* receptor may elicit altered physiological states by binding other cellular receptors. Exogenous chemicals that interact with cellular steroid receptors to initiate gene expression typically regulated by endogenous hormones have been broadly characterised as xenoestrogens. Oestrogen mimetic activity has been demonstrated for a variety of environmentally encountered

chemicals, including chlorinated pesticides, insecticides and herbicides, endosulfans, the polychlorinated biphenyls, dibenzofurans and dioxins, octylphenol and nonylphenol, detergents, phytoestrogens and metabolic products of this diverse group of chemicals. Other than the apparent requirement for a phenolic ring, the array of compounds found to have estrogenic activity may differ widely, pointing out the difficulty in using structure/function analyses to predict the oestrogenicity of chemicals (Duax and Griffin, 1985; Müller *et al.*, 1995).

Endogenous steroids may either maintain or alter states of physiological function by interaction with a variety of nuclear receptors. They bind receptors with high affinity and specificity, forming ligand-dependent complexes that initiate gene transcription in cells that express the appropriate ligand-binding receptor. Xenobiotic chemicals which bind steroidal receptors typically stimulate the same responses in cells as natural ligands, but, in pure form, usually have significantly less (up to 10,000-fold) affinity of binding to steroid receptors (Arnold *et al.*, 1996). Pollutant chemicals, however, almost never exist in pure form, and synergistic effects of complex pollutant mixtures result in oestrogen receptor binding affinities which may be multiple log units higher than those of individual chemical components (Arnold *et al.*, 1996).

Xenoestrogenic pollutants, either individually or as components of complex mixtures, have endocrine disruptive activities known to adversely affect physiological homeostasis, including reproduction, foetal development, sex determination and immune system function, and have been proposed to be etiologic factors in hormonally associated cancer initiation. Xenobiotic-altered reproductive capacities can potentially be related to sex determination abnormalities during foetal development or to physiological effects on adult organisms. As an example of altered physiology, the quality of human semen is reported to have declined over the last twenty years along with a decline in the sperm counts of males (Carlsen *et al.*, 1992; Sharp and Skakkebaek, 1993). Xenobiotic-associated feminisation has also been reported as a mechanism altering normal sex determination in birds, fish, shellfish, non-human mammals and reptiles (Colborn *et al.*, 1993; Crews *et al.*, 1994; 1995).

Foetal development is apparently altered in both subtle and overt ways by exposure to endocrine-disrupting xenobiotic chemicals. This may result in altered structure and function of reproductive tract tissues (Iguchi *et al.*, 1995; Jones and Hajek, 1995) and both skeletal and soft-tissue morphological abnormalities (Migliaccio *et al.*, 1995). These abnormalities appear related to the agonistic or antagonistic regulatory effects of xenobiotics on growth factors, and point out that interactions of xenobiotics with a variety of cellular receptors in addition to the *Ah* and steroid receptors may adversely impact foetal and neonatal development (Birnbaum, 1995).

The pleiomorphic expression of immune system dysfunction in response to xenobiotic exposure has puzzled investigators for years. The variety of immune system responses to xenobiotics is apparently due to the degree to which the immune and endocrine systems are intimately interactive, with many steroids being exquisite regulators of immune system function (Ralston *et al.*, 1990; Laird *et al.*, 1993). Different isomers and congeners of xenobiotics such as dioxin and PCBs can have strikingly different actions to either elicit or inhibit expression of different cytokine and growth factor genes (Safe, 1994). The end result of immune system dysregulation from xenobiotic altered expression of different cytokines or growth factors remains, however, much the same. Animals exhibit increased susceptibility to viral, bacterial, fungal and parasitic infections, and may present a wide variety of individual and species responses.

Chronic TCDD toxicity in rodents is evidenced primarily as hepatic dysfunction, whereas chronic administration of low TCDD concentrations to monkeys leads to skin abnormalities,

gastritis and gastric ulcers, and to immune dysfunction as an apparent consequence of hypocellular bone marrow resulting in decreased levels of circulating myeloid and lymphoid cells. Acute and subacute TCDD toxicity in the same animals leads to thymic involution and a variety of immunological defects resulting in failure of the immune system. Thus, the effects of chronic low-level exposure to TCDD as a model organochlorine may differ from those observed in acutely exposed animals, and species-specific effects of chronic exposure to low levels of organochlorines may occur.

Determination of hydrocarbon pollutants in marine mammals

Halogenated hydrocarbons, particularly the PCBs, have been reported as residues in cetaceans from virtually all of the world's oceans. PCBs and related chemicals are found in essentially all high triglyceride deposition tissues of cetaceans, including blubber, mammary glands and melon fat as well as in serum, brain, spleen, liver, muscle and kidney of marine mammals (Aguilar, 1985).

PCBs are adsorbed onto high lipid content membranes of phytoplankton, and are subsequently internalised (Broman et al., 1992) and sequestered in intracellular lipids until the organism is consumed by a higher order species. Tanabe (1988) reported that organochlorine bioaccumulation factors between ten thousand and one million times the source concentration may occur in specific species, and that these values are dependent on body fat content and the trophic level occupied by the organism. While the total oceanic content of PCBs has been estimated at 1 part per trillion, specific areas such as the North Atlantic have significantly higher PCB concentrations. A million-fold bioaccumulation of 1 part per trillion would yield only a few parts per million for high lipid tissues, whereas higher organochlorine concentrations have been reported for many cetacean species. For example, harbour porpoises and bottlenose dolphins stranded on the coast of Scotland were reported to have about 23ppm PCBs, with 10.2ppm of DDT (Wells et al., 1994). Striped dolphins that died in the western Mediterranean epizootic exhibited a high of 3,000ppm PCBs, with the majority of animals evaluated showing PCB levels between 500 and 2,000ppm in blubber (Aguilar and Borrell, 1994). Elevated levels of PCBs would be expected for near-shore cetaceans that exhibit high fat per total body weight ratios, are long lived and are upper trophic level carnivores (Honda et al., 1992). However, the levels reported by Wells et al. (1994), Aguilar and Borrell (1994) and de Kock et al. (1994) are clearly excessive and indicative of both high pollutant concentrations in resident waters and efficient bioaccumulation. Pelagic cetaceans inhabit relatively pristine waters and feed lower on the food chain tend to exhibit lower tissue residue levels of organochlorines (O'Shea and Brownell, 1994).

The consistent findings of organochlorine residues in tissues from cetaceans in polluted and pristine waters have resulted in the need to correlate data on residue levels with the health of the animals. This is particularly true in those instances where the highly toxic non-ortho coplanar congeners, such as IUPAC 77, 126 and 169, have been found (Falandysz et al., 1994). Kuiken et al. (1994) were unable to correlate PCB levels in tissues of harbour porpoises (Phocoena phocoena) with healthy animals that died of trauma as opposed to animals that died of infectious disease of some sort. Kannan et al. (1993) completed isomer-specific analyses of PCBs from striped dolphins in the western Mediterranean epizootic, reporting concentrations ranging from 94-670µg/g (wet weight). They reported that TCDD toxic equivalents for non-, mono- and di-ortho congeners were several times higher than is typical for humans or other marine mammals, and that, even though non-ortho congeners are more toxic, mono-ortho congeners contributed higher levels of TCDD toxic equivalents in these animals than did non-ortho congeners. Aguilar and Borrell (1994) proposed that the high levels of PCBs in the liver, as opposed to the blubber, of animals in

the striped dolphin epizootic suggest that blubber lipid reserves may have been mobilised resulting in elevated serum levels of PCBs with deposition of mobilised PCBs in the liver. However, they were unable to determine whether this led to any pathological effect in the animals that died during the epizootic. Kannan *et al* (1993), however, proposed that the ratio of IUPAC 169/126 in the epizootic animals suggested the likelihood of a significant induction of mixed function oxidase enzymes, a factor that might be indicative of physiological stress.

While induced cytochrome P450 levels and elevated metabolism of hydrocarbons had been previously shown in dolphin tissues, the presence of a TCDD-binding *Ah* receptor protein was first demonstrated in bottlenose dolphin epithelial cells (CDK cell line) established from foetal dolphin kidney tissue (Carvan *et al.*, 1994). TCDD-induced CDK cells metabolised benzo (a) pyrene (BP) *in vitro* to form BP-DNA adducts capable of initiating DNA excision repair (Carvan *et al.*, 1995). Cellular proliferation induced with 10^{-8} M TCDD and treated with 10^{-7}M BP was reduced to 50% of control levels. Proliferation in non-TCDD-induced cells treated with 10^{-5}M BP as both an inducing agent and carcinogen source was approximately 10-fold lower than the control. The BP-decreased proliferative capacity was effectively eliminated by the inhibition of cytochrome P450 induction with α-naphthoflavone. These data suggest the possibility that *Ah* receptor-mediated induction of gene expression in dolphin cells could be associated with cytotoxic phenomena *in vitro*.

Pinnipeds

Seals, sea lions, fur seals and walruses have been found to contain variable tissue concentrations of a number of persistent organochlorines, including PCBs, the halogenated hydrocarbon pesticides and their variety of metabolites (e.g., DDT, dichlorophenyldichloroethane, hexachlorocyclohexane, chlordane and its metabolites, and certain of the cyclodienes such as dieldrin and aldrin), along with complex mixtures of other lipid soluble hydrocarbons (e.g. Hutchinson and Simmonds, 1994).

The variety of contaminant analyses reported from animals differing in species, age, gender, location, times of year, time since death occurred, states of lactation and states of nutrition, coupled with difficulties in identification of specific chemicals and different congeners of the same class of chemicals, has made it very difficult to compile data useful for a determination of the correlation between chemicals and the onset of contaminant associated diseases. For instance, Duinker *et al.* (1988) reported the number of chlorine atoms of significant PCB congeners to range from 3-7, stating that the composition of PCB mixtures in tissue residues cannot easily be accurately determined in terms of a single formulation (e.g. Aroclor vs Clophen). Determination of specific congeners of PCBs is extremely important since they may differ widely in toxicity dependent on the number and location of chlorine groups (Safe, 1984; 1994; Harper *et al.*, 1995), and since complex mixtures of the chemicals may or may not be additive in their immunosuppressive characteristics (Harper *et al.*, 1995) and distribution patterns (Duinker *et al.*, 1988). In addition, they may exhibit interspecies differences in uptake, transformation and excretion patterns that will continue to make it difficult to evaluate mixtures of compounds as to their effects on animals.

Many of the early studies of organochlorines in pinniped tissues expressed chemical concentrations in terms of wet tissue weights, with the greatest concentrations of organics found in the blubber. The reported chemical concentrations differ dramatically when expressed in terms of extractable lipids. This is not surprising, considering that blubber organochlorine residues account for approximately 98% of total body residues. Hutchinson and Simmonds (1994) point out that a major problem in determining whether

organochlorines are exhibiting significant increases in tissue residues lies in the historical absence of standardised sampling and analysis methods. The recent adoption of more standardised analytical methods and the availability of more highly purified chemical standards has resolved many of the past problems inherent in residue analysis. However, complex mixtures of organics and their metabolites found in pinniped tissues continue to be difficult to determine, and to be dependent on environmental persistence and mobility, the variety of congeners and congener toxicity of the chemicals, the age, gender, state of nutrition, health of the animal and species capacity to be induced for enzymes that conjugate or metabolise residue chemicals.

The relationship between exposure to specific chemicals or mixtures of chemicals in the environment and health has been difficult to establish. Developmental abnormalities, evidenced as skeletal defects, in ringed seals, harbour seals and grey seals from areas of the Baltic Sea were reported to have increased after 1955, correlating with the increase in tissue organohalogen levels (Zakharov and Yablokov, 1990; Bergman *et al.*, 1992; Mortensen *et al.*, 1992). These defects increased in occurrence during a time when environmental organochlorine concentrations were increasing and the seal population was decreasing, and were proposed to be associated with altered endocrine function in pregnant females. Abnormalities in foetal development have also been reported to be potentially associated with blockage of the uterus in seals (Helle, 1980; Reijnders, 1994), with the suggestion by Reijnders (1994) that perturbations in the endocrine system caused by PCBs could be a cause of foetal abnormalities, foetal resorption or uterine pathologies. All of these could potentially be indicative of a broader state of reduced reproductive efficiency in animals exposed to specific endocrine interactive organochlorines; however, alternative causes of these abnormalities cannot be excluded. Potential organochlorine-associated reproductive dysfunction has also been reported for sea lions in California, where tissue concentrations of PCBs in prematurely born pups were from 200% to 800% higher than in pups born full-term (De Long *et al.*, 1973). Although complicating factors, such as differences in metal concentrations and the potential presence of micro-organisms capable of causing spontaneous abortion, clouded the initial conclusions of the study, the presence of elevated PCB concentrations could not be ruled out as being causative to the reproductive dysfunction (Addison, 1989).

Endocrine alterations induce reproductive and developmental disorders. They have long been associated with known states of immune suppression in humans and experimental laboratory animals (Margolick, 1992; Newcombe, 1992) leading to increased indices of infectious disease. The epizootic-associated deaths of large numbers of marine mammals, along with recently developed data providing potential understanding of the mechanisms by which organochlorines might impact endocrine function, have generated considerable interest in the possible association between pollutant-initiated immune suppression and infectious diseases in marine mammals.

Large scale mortalities of marine mammals, the vast majority of which have occurred in modern times, include: harbour seals in Iceland in 1918, and in 1979 and 1980 in the northeastern United States; Bering Strait walruses in 1978; Baikal seals in 1987; bottlenose dolphins on the east coast of the United States in 1987 and 1988; bottlenose dolphins in the Gulf of Mexico in 1990, 1992, 1993 and 1994; and striped dolphins in the Mediterranean and Aegean Seas in 1990, 1991 and 1992. In most of these, useful data were obtained from some of the animals pertaining to age, gender, body condition, overt pathologies and tissue burdens of organohalogens. However, the variable states of decomposition resulted in some difficulties in evaluation of chemical residues, viral and immune function, leaving open the question(s) regarding underlying causes of death in virtually all of the animals that died in even the most recent of epizootics. Nevertheless, immune system dysfunction resulting from

organochlorine exposure has been widely demonstrated in laboratory animals (Safe, 1984; 1990; Harper *et al.*, 1995), and there is evidence suggesting that impaired immune function in pinnipeds with elevated tissue residues of organochlorines may be associated with increased indices of infectious disease (Brouwer *et al.*, 1989).

Mechanisms of organohalogen and hydrocarbon toxicity

Environmental exposure of animals to TCDD and related chemicals, including a variety of PCB congeners, is associated with a number of toxic physiological responses, including immunosuppression, thymic involution, weight loss, hepatotoxicity, porphyria, severe dermal lesions, foetotoxicity and severe birth defects, and a dramatic wasting syndrome ultimately leading to death (Safe, 1986; 1990; 1994). These responses differ dramatically between inbred strains of animals, with LD_{50} values between 0.6-2.0 µg/kg for guinea pigs and 1,157-5,000 µg/kg for hamsters (88), and have not been defined for any of the marine mammals. Toxic equivalency factors (TEF) are available for many of the more active isomers and congeners of TCDD, PCDD and related compounds, and are expressed as potential measures of toxicity in laboratory animals relative to the effects of TCDD (Goldstein and Safe, 1989; Safe, 1990; Harper *et al.*, 1995).

The variety of animal responses to toxic hydrocarbons, including some of the organochlorines, is related to the fact that these chemicals may induce expression of a variety of enzyme systems (Poland and Knutson, 1982; Safe, 1984; Whitlock, 1986; 1987), including the phase I cytochrome P450 enzymes such as ethoxyresorufin O-deethylase (EROD), and the phase II enzymes such as glutathione S-transferase (Tables 1 and 2). The mechanisms by which polycyclic aromatic hydrocarbons induce P450 expression, and the subsequent metabolism of PAHs by P450-associated enzymes to produce reactive compounds capable of causing macromolecular adducts and cell damage has been extensively investigated. In contrast, the precise mechanisms by which organochlorines exert cytotoxic effects are not well understood. Some of the organochlorines also act by binding cellular receptors with subsequent altered expression of both cytochrome P450 and non-P450 genes. Altered gene expression may, in turn, alter critical cellular processes

Table 1

Selected Inducible Phase I Enzyme Systems.

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*Cytochrome P450 (multiple families and enzymes within families)
*Nicotinamide adenine dinucleotide-b5 reductase
*Nicotinamide adenine dinucleotide-P450 reductase
*Epoxide hydrolase
*Esterases
*Aldehyde/ketone reductase
*Monoamine oxidase
*Diamine oxidase (histaminase)
Amidohydrolases (amidases)
Amine oxidase (flavin-containing monooxygenase)
Alcohol oxidoreductase (alcohol dehydrogenase)
Aldehyde oxidoreductase (aldehyde dehydrogenase)
Xanthine oxidase
Aldehyde oxidoreductase (aldehyde oxidase)

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*Enzyme activity has been measured in lymphocytes.

Table 2

Selected Phase II Detoxification Enzymes.

*N-acetyl transferase
*Thiopurine methyltransferase
*Glutathione-S-transferase
*Catechol-O-methyltransferase
*Uridine diphosphate glucuronyl transferase
Cytoplasmic sulfotransferases
Amino acid conjugases (glutamine, cysteine)
Thio methyltransferase

*Enzyme activity has been measured in lymphocytes.

resulting in the onset of toxic phenomena. Not all organochlorines bind the cellular receptors, and exert cytotoxic effects by other mechanisms.

Recent data show that the characteristic interaction of some organochlorines with normal cells is dependent on the initial binding to cellular receptors. These may include members of the steroid receptor superfamily (Dolwick *et al.*, 1993b) and at least one member of the basic helix-loop-helix (bHLH) superfamily of DNA binding proteins, the *Ah* receptor (Poellinger, 1995). The protein product specified by the *Ah* receptor gene, AhR, is one of the ligand-activated transcription factors that belongs to the bHLH family of DNA interactive proteins. These are activated by binding to either exogenous anthropogenic ligands such as dioxin (saturable high affinity binding, K_d of about 1 nM), exogenous dietary ligands such as indolo[3,2-*b*]carbazole, or to endogenous ligands, which have not been identified. Ligand binding is followed by loss of the chaperone heat shock protein, hsp90, dimerisation of the AhR-ligand complex with the nuclear transport protein, Arnt (Li *et al.*, 1994; Whitelaw *et al.*,
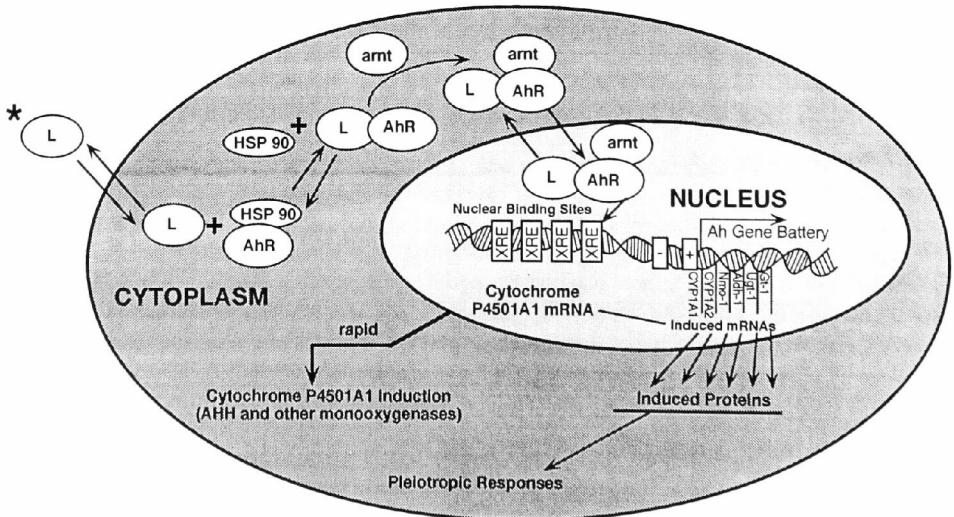
Fig. 3. A depiction of the sequential mechanisms by which AhR-binding ligands interact with the cell to initiate expression of a variety of different genes. The ligand, TCDD, PCBs, benzo(a)pyrene, 3-methylcholanthrene, or any number of other AhR-interactive agents, binds AhR, the chaperone protein HSP-90 dissociated from the AhR-ligand complex, the aryl hydro-carbon receptor nuclear transport protein, arnt, binds the complex as it is transported into the nucleus, and the AhR-ligand complex binds to XRE (DRE) sites, allowing transcription of downstream genes to give variety of physiological responses.

1994), and transport of the receptor-ligand complex into the nucleus (Fig. 4). The intranuclear AhR-ligand/Arnt complex interacts with xenobiotic response elements of DNA, XRE (also called dioxin response elements, DRE), resulting in the initiation of mRNA transcription and the synthesis of a variety of gene products, including phase I enzymes such as the CYP isoforms. In addition, AhR-ligand binding to DNA appears to regulate expression of a number of growth modulatory genes, such as plasminogen activator inhibitor-2 and interleukin 1-β (Sutter *et al.*, 1991). Dioxin binding to AhR in mice has been linked to expression of growth modulatory genes that may be important in the development of AhR-mediated toxic phenomena, including thymic involution and cleft palate formation (Poland and Knutson, 1982; Safe, 1990). AhR is the only ligand activated bHLH transcription factor known at this time; however, other bHLH type receptor proteins are known to regulate cell type-specific transcription, the initiation of mitosis and cell proliferation, and cell transformation (Prendergast and Ziff, 1992). These include the myc oncoproteins, and drosophila genes regulating neurogenesis, mesoderm formation, sex determination, formation of the peripheral nervous system (Jan and Jan, 1994) and early development of olfactory and autonomic neurons (Guillemot *et al.*, 1993).

The *Ah* receptor gene is constitutively expressed in the cells of all normal animals and in a variety of tissues within each animal (Dolwick *et al.*, 1993a), and expression of the *Ah* receptor gene is essential for the health of the animal. Genetically engineered mice (knockout mice) which lack production of AhR (*Ah$^{-/-}$*) either die at birth or live a short life marred by liver damage and immune dysfunction resulting in repeated infections (Fernandez-Salguero *et al.*, 1995). *Ah$^{-/-}$* mice that live past birth never develop normal liver function and are subject to progressive liver damage leading to fibrosis and an early death. Birnbaum and co-workers (Abbott *et al.*, 1991) speculate that a yet unknown endogenous ligand for AhR plays a critical role in foetal development and homeostasis in mice. This is seen in the fact that *Ah$^{-/-}$* mice lacking the AhR protein also lack the capacity to initiate normal immune system function when faced with foreign antigens against which an immune response would be initiated in normal animals, apparently due to the inability to initiate T cell proliferative responses. The interaction of dioxins and PCBs to produce the sequel of toxic effects seen in animals (Hebert *et al.*, 1990; De Vito *et al.*, 1994), and the inability of *Ah$^{-/-}$* mice to develop normally clearly shows a role for AhR in the regulation of a variety of normal physiological functions in animals. Birnbaum has speculated that the orchestrated interaction of the AhR with its normal endogenous ligand(s) may be significantly perturbed by the presence of exogenous ligands capable of binding the AhR protein with high specificity, and that exogenous ligand binding to AhR may induce the inappropriate expression of a variety of genes, the products of which interfere with normal cell development and function. While the AhR-binding xenobiotics are toxic to adult animals, leading to the loss of immune function associated with thymic atrophy and hypocellularity of bone marrow, they can be devastating in prenatal and neonatal animals, leading to a broad spectrum of developmental defects in addition to the inability to develop normal immune system function. Again, the cellular effects initiated by organochlorine binding to AhR are species specific and toxic effects are not initiated by organochlorines in all species.

These very recent data provide a foundation for understanding the possible mechanisms by which environmental pollutants interact with marine mammals to decrease their immune function and increase their susceptibility to infectious diseases. This initial understanding of the potential mechanisms of immunotoxicity was developed in laboratory animals and must be evaluated to demonstrate applicability of the mechanism to marine mammals. Expression of the *Ah* receptor, binding of the AhR protein to dioxin, and increased cytochrome P450-associated metabolism of hydrocarbons in dioxin-induced cells has been demonstrated in bottlenose dolphin cells *in vitro*, and has been indirectly shown in white whales and other

marine animals. These data provide a starting point for investigations into the cellular mechanisms by which organochlorines and related compounds initiate immune dysfunction in marine mammals.

BASIC IMMUNOLOGY: AN UPDATE FOR THE NON-SPECIALIST

Introduction

If an animal is to survive, it must be able to exclude and/or defend against invading pathogens. There are two major components of the immunological defensive response to micro-organism invaders, each effecting a defence against one of the two relatively distinct categories of infectious agents. The antibody response is directed primarily against exogenous antigens, such as bacterial proteins and polysaccharides, while the cell-mediated response is directed primarily against antigens from transformed cells or endogenous viral antigens that originate in virus infected cells.

When a foreign antigen enters an animal, it is initially engulfed, hydrolytically processed and presented so that it can be recognised as non-self. This information is transmitted either to the antibody-forming system or to the cell-mediated system. These respond by producing specific antibodies and/or activated cells that function to eliminate or neutralise the foreign antigen. The immune system also has a memory component so that when it encounters the same antigen again, its response will be faster and more efficient.

The immune system thus has four basic components. It needs (i) a method of trapping and processing antigens; (ii) a mechanism for recognising and stimulating a response to specific antigens; (iii) cells that either produce antibodies specific for reactive domains on the antigen or that participate in cell-mediated immune attack on the antigen; and (iv) memory cells capable of reacting rapidly to the same antigen if it is encountered again. Each component is associated with specific cell types. Antigens are engulfed, processed and eventually eliminated by combinations of cells that may include macrophages, dendritic cells and lymphocytes. Lymphocytes have specific receptors that interact with and respond to foreign proteins presented as processed antigenic peptides. The memory function of the immune system is resident in specific lymphocytes capable of initiating a secondary immune response. Cell-mediated responses are initiated by T lymphocytes, while antibody synthesis and secretion is a function of B lymphocytes.

Antigen processing

There are several steps in exogenous antigen capture and processing by macrophages. The antigen must initially be phagocytised and incorporated into phagosomes (Gerlier and Rabourdin-Combe, 1989). The phagosomes are fused with cytoplasmic lysosomes to form endosomes containing acidic proteases, which degrade exogenous proteins into fragments about 10-20 amino acids long. Endosomes containing these antigenic peptide fragments then fuse with other endosomes carrying specialised type II major histocompatibility complex proteins (MHC type II) which function on the cell membrane as a type of receptor. As the antigenic peptide fragments and MHC II proteins move to the cell surface, the non-self peptide fragments become aligned with a groove on the MHC II molecule and bind there. The endosome vesicle fuses with the cytoplasmic membrane and the MHC II/peptide complex is oriented on the cell surface in a form that can be recognised by receptors on T cells. T cells which bind the MHC II complex and have a unique receptor capable of interacting with the antigenic peptide are triggered into responding by binding of their receptor with the antigen. Macrophages regulate the dose of antigen presented on their plasma membrane along with MHC II proteins, and so prevent the inappropriate

development of tolerance to antigens. If an antigen is presented to T cells without being linked to an MHC II molecule, the cells may be turned off and tolerance to the antigen may result.

Receptors of the immunoglobulin superfamily

The key proteins having domains which serve as receptors that bind antigenic peptide fragments belong to the immunoglobulin superfamily, which includes many functionally different proteins (Burton, 1990; Cambier and Campbell, 1992). Some have multiple immunoglobulin domains, others have only a single domain. The globulins with multiple domains include antibody molecules (immunoglobulins), T-cell antigen receptors (TCR) and MHC class I and II molecules. Common features of the immunoglobulin superfamily are that all members are involved in binding to other molecules, most are found on cell surfaces and none have enzymatic activity. In many cases, cell interactions are mediated by two different members of the immunoglobulin superfamily, for example, TCR and MHC molecules mediate interaction between antigen presenting cells and T cells.

Immunoglobulins may serve as B cell receptors or as antibodies
The macrophage also secretes a variety of cytokines, including interleukin 1, or IL-1, which binds to receptors on CD4+T cells, called T helper or Th cells. Activated CD4+T cells then secrete a milieu of lymphokines, including IL-2, which interacts with receptors on a number of different cells, including CD8+T cells, B cells, and, in an autocrine or self-activating mode, CD4+T cells. B cells are capable of synthesising immunoglobulins that are unique to each B cell type. The globulin genes have gone through a splicing process during maturation of the cell that results in all B cells having a different variable region on their membrane bound globulins, and in each cell producing only one type of globulin variable region. When that variable globulin domain is capable of interacting with the peptide presented by MHC II. the B cells bind the MHC II-peptide complex and are stimulated via one or more signal transduction mechanisms to enter mitosis and begin formation of B cell clones. Cells of the clone, a group of cells derived from a common progenitor B cell, further mature and begin secreting immunoglobulins, each of which has an identical variable domain capable of interacting with the antigenic peptide presented to the progenitor B cell by the macrophage.

Processed antigens bind special receptors called MHC molecules
MHC groups I and II are complex proteins that are uniquely polymorphic. That is, they differ in their unique combination of different proteins between almost every individual, and their complex structure determines which antigenic peptides can bind to the MHC. MHC molecules determine whether an individual can respond to a specific antigen, and MHC binding is essential for initiating an immune response against a specific peptide region, called an epitope. If an antigenic peptide does not bind to an MHC molecule, no immune response can be initiated against that peptide. For this reason, the genes determining MHC molecules are called 'immune response genes'. These genes dictate resistance or susceptibility to many diseases in animals.

   MHC Class II molecules are glycoproteins consisting of two polypeptide chains called α and β. Two domains of each peptide chain ($\alpha 1$ and $\beta 1$) fold together to form an open-ended groove which functions as an antigen binding site. The overall shape of the MHC II groove is determined by conserved peptides of the α and β domains while the polymorphic residues determine the precise shape of the groove. The MHC groove can interact with a peptide of

12-24 amino acids as a straight chain that projects out of both ends. The individual shape of specific MHC determines the ability of each class II molecule to bind antigen fragments, and thus dictates the ability of an animal to respond to a specific antigenic peptide. MHC II are found only on antigen presenting cells, whereas all nucleated cells of an organism contain MHC I proteins.

A second type of MHC molecule involved in binding endogenous antigens, tumour cell or viral antigens generated within the body, is called MHC class I. MHC class I molecules bind peptides typically originating within the cell. The processing of these peptides is very different from those associated with class II molecules. Normal cells continually break down within the body and recycle proteins. During this process abnormal proteins are removed, regulatory peptides are not allowed to accumulate and amino acids are made available for other purposes. Newly formed proteins in virus-infected cells are hydrolysed by large proteolytic complexes called proteasomes (Goldberg and Rock, 1992). As a protein is degraded, peptides are rescued from further breakdown by attachment to transporter proteins that carry protein fragments from the cytoplasm to the endoplasmic reticulum, where MHC class I molecules are synthesised and assembled around the peptides which lie within the MHC groove. MHC class I molecules carry peptides to the cell surface for recognition. When the MHC I/peptide complex reaches the cell surface, the bound peptides are displayed and are available for interaction with specific cells having receptors for which the MHC-associated peptides have interactive specificity.

T cells recognise antigens through a special receptor

T cells will only respond to antigens attached to an MHC molecule if those antigens are recognised by the T cell antigen receptor, or TCR (Hodgkin and Kehry, 1992). Each T cell has between 10,000 and 20,000 identical TCRs on its surface. These TCRs are protein complexes closely associated with an additional protein designated as either CD4 or CD8. A CD4 or CD8 molecule is required in order to link the T cell to the MHC molecule on the antigen-presenting cell. CD4 binds to MHC class II molecules while CD8 binds to MHC class I molecules. CD4+ T cells bind and respond to processed exogenous bacterial and parasitic antigens, while CD8+ T cells bind and respond to processed endogenous viral and tumour antigens.

The TCR is a complex structure, as one would expect for a critical antigen-binding receptor. It consists of two major parts, one that confers binding specificity to antigens and MHC, and a second that is involved in signal transduction mechanisms that initiate signals from the receptor protein to the T cell, triggering intracellular responses. TCR are always associated with a set of invariant glycoproteins collectively called CD3. These CD3 glycoproteins are the signal-transducing parts of the TCR receptor. They do not function simply as an on/off switch for mitosis, but are linked to at least three different signal transduction pathways (Weaver and Unanue, 1990). Depending on which combination of pathways is triggered, the T cell may divide and/or secrete proteins called cytokines. If the receptor is only partially triggered then the T cell may be turned off. This is a protective device to ensure that T cells do not respond inappropriately to self antigens.

Two types of mammalian TCR have been identified. One has two component peptide chains called gamma and delta. The other consists of two different peptide chains, alpha and beta. In humans, mice and (probably) most non-ruminants, between one and ten percent of T cells carry gamma and delta receptors. The remaining 90-99% of T cells in these species carry alpha and beta receptors. In artiodactyls, in contrast, T cells with gamma and delta TCR can account for as much as 60% of the total T cell population. The four T cell receptor peptide chains are similar in structure although their molecular weights differ. Each TCR

chain is divided into four well defined domains. The C-terminal end of each chain is attached to the T cell membrane and has a constant amino acid sequence, thus, it is called the constant region (C-region). The TCR domain exposed at the cell surface has a highly variable sequence and is therefore called the variable region (V-region). The TCR protein functions essentially as an immunoglobulin with a constant domain imbedded within the cytoplasmic membrane as an effector oriented towards the signal transduction target. When two cytotoxic cells compete for binding to the same MHC I and antigenic peptide only one is triggered, perhaps the one with highest binding affinity for the antigen.

Summary

In a model immune response to bacterial invasion, macrophages engulf a bacterium and hydrolytically degrade the bacterial proteins, presenting them on the cytoplasmic membrane in association with the MHC II complex. The macrophage also secretes a variety of cytokines, including interleukin 1, or IL-1, which binds to receptors on CD4+ T cells, called T helper or Th cells. Activated CD4+ T cells then secrete IL-2, which interacts with receptors on a variety of cells, including CD8+ T cells, B cells, and, in an autocrine or self-activating mode, CD4+ T cells. Activated B cells that produce a variable globulin domain capable of interacting with the peptide presented by MHC II bind the MHC II-peptide complex and are stimulated via one or more signal transduction mechanisms to enter mitosis and begin formation of a B cell clone. Cells of the clone, a group of cells derived from a common progenitor B cell, mature and begin secreting immunoglobulins, each of which has an identical variable domain capable of interacting with the antigenic peptide presented to the progenitor B cell by the macrophage.

If the immune response is triggered by endogenous antigens such as those on a virally infected cell or a transformed (neoplastic) cell, the macrophage engulfs the infected cell, digests the proteins to peptides and presents the antigenic non-self peptides in concert with an MHC I complex. Again, the macrophage secretes IL-1, which stimulates CD4+ T cells to secrete IL-2. When IL-2 binds receptors on CD8+ T cells (cytotoxic T lymphocytes) the CD8+ T cells recognise the MHC I complex and bind to it. Those CD8+ cells that have a globulin receptor, TCR, capable of responding to the antigenic peptide are stimulated to initiate mitosis and the cytotoxic T cells form a clone, each cell of which is capable, by virtue of its globulin receptor, of binding to cells in the body having virally encoded or transformed specific membrane proteins like the one the macrophage presented. When cytotoxic T cells bind to cells expressing the viral or transformed cell proteins they mount an enzymatic attack on the cell that kills it (O'Rourke and Mescher, 1993).

Lymphoid organs and cells of the immune system are strategically located to monitor physiological portals of entry and may be among the first cells to participate in the absorption, distribution and biotransformation of xenobiotic compounds entering an animal. In mammals, ingested fat-soluble chemicals are taken up into the lacteal absorption from the gut and transit the mesenteric lymphatic drainage to the vascular circulation via the thoracic duct, bypassing the liver (Yoo *et al.*, 1984; Busbee *et al.*, 1985; Wilson *et al.*, 1985). Therefore, fat soluble chemicals in the diet incorporated into chylomicrons are peripherally distributed prior to encountering hepatic detoxification mechanisms, with potential exposure of essentially all peripheral organs and cells to unmetabolised fat-soluble hydrocarbons. Immune function cells in the lymphatic organ-rich thoracic duct are directly exposed to extremely high levels of fat-soluble chemicals. Cells of myeloid and lymphoid origin constitute the first line of defence against microbial invaders, and conduct constant immunosurveillance against cells showing antigens indicative of virus infections or of neoplastically transformed cells (Newcombe, 1992). Anything capable of interfering with

the complex physiological processes required for myeloid cell function and activation of either the antibody-associated or cell-mediated lymphoid responses could ultimately lead to the loss of protection against microbial pathogens and neoplastic cells.

IMMUNOLOGY AS A TOOL FOR ASSESSING MARINE MAMMAL HEALTH

Practical limitations are imposed upon establishment of unequivocal associations between environmental contaminants and marine mammal health. Marine mammals are protected species and thus fulfilling Koch's postulates relative to infectious agents and potential toxic compounds is not realistic (e.g. see Mims and White, 1984). Furthermore, the ability to sample individual animals over time in their natural environment is not an easy task and the use of invasive techniques to obtain samples is probably not appropriate. Development of quantitative and qualitative techniques to measure immune system function represents the most viable and sensitive monitor of marine mammal health. The characterisation of a given animals' immune system components will provide a sensitive indicator of perturbations to the system, provided sufficient age, sex and reproductive status baseline data are available for the species. Blood of such species may be relatively easily obtained in sufficient quantities by non-invasive means (venipuncture via the fluke in cetacean species or accessing a venous sinus in pinnipeds) with the resulting serum, plasma and leukocytes providing a window for assessing immune system status.

Knowledge about the immune system of marine mammals is limited. Normal ranges of total white blood cell (WBC) counts, and associated differentials, serum immunoglobulin (Ig) levels and limited blood chemistry values have been reported for some species as summarised by Bossart and Dierauf (1990) and Kennedy-Stoskopf (1990). However, the number of animals sampled is often quite small and minimal data available relate to the influence of animal age and reproductive status on the reported values. Such measurements of immune system components are valuable to assess an animal's health and provide gross insight into existing potential disease (Bossart and Dierauf, 1990). The measurement of antigen-specific antibody, as a measure of current and past exposure to infectious agents, can be realised using plasma, or preferably serum, in standard serologic assays. Serologic assays such as enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA) require secondary reagents for identifying the presence of specific antibody. For the large part, anti-Ig reagents readily available for human, marine and domestic animal species have not been adequately characterised as to their specificity for equivalent Ig molecules in marine mammals. While some cross-species reactivity has been documented (Nash and Mach, 1971; Cavagnolo and Vedros, 1978; Cavagnolo, 1979), it is desirable to establish reagents specifically for use with each marine mammal species. The use of protein A (Ross *et al.*, 1993) or G (conjugated to a detection system) for detection of marine mammal Ig can serve as a secondary reagent; however, efficacy of such binding may not be uniform between animal species and antibody isotypes. The preferable reagent for routine serologic analysis would be species-specific anti-heavy and –light chain Ig such that all antibody isotypes would be identified. Such reagents, anti-harbour seal Ig (Carter *et al.*, 1990) and anti-dolphin Ig (Romano *et al.*, 1992), have undoubtedly been prepared by multiple investigators (our laboratory has developed such reagents for dolphins, harbour seals and sea otters) but are not available on a commercial basis. Furthermore, polyclonal (Kennedy-Stoskopf, 1990) or monoclonal antibodies with Ig heavy chain specificities are also of value; identification of high titer antigen-specific IgM and low titer IgG would be indicative of an active infection and preclude the necessity for paired serum samples (samples obtained over a period of time) for demonstrating a rise in antibody titer to a given antigen.

Identification of immune system dysfunction as a mechanism of assessing marine mammal health using serologic data, gross haematology and clinical chemistries are probably of limited value. Acquired immunodeficiency, as opposed to malfunction at the inherited or genetic level, has classically been identified by measurement of lymphocyte function employing a lymphocyte blastogenesis assay on peripheral blood mononuclear leukocytes. Such assays measure the ability of lymphocytes to proliferate following polyclonal stimulation with mitogens; proliferation is typically detected by incorporation of ^{3}H-thymidine. Lymphocyte blastogenesis data have been reported for bottlenose dolphins (Colgrove, 1978; Lahvis *et al.*, 1993) and harbour seals (De Swart *et al.*, 1993; Ross *et al.*, 1993), using a variety of mitogens including phytohemagglutinin (PHA), concanavalin A (ConA), pokeweed mitogen (PWM) and bacterial lipopolysaccharide (LPS). Antigen-specific induction of lymphocyte proliferation (blastogenesis) and delayed-type hypersensitivity (DTH skin test) have been reported in harbour seals following immunisation (De Swart *et al.*, 1993). However, such antigen-specific analysis is not realistic when assessing the immunocompetence of free-ranging animals. While blastogenesis assays provide gross insight into lymphocyte function and can identify animals with substantial immunosuppression, they have inherent disadvantages including: (i) poor reproducibility (especially between laboratories); (ii) the lymphocyte subset(s) induced to proliferate are unknown; and (iii) the counts/minute (CPM) or stimulation index (SI) should probably not be used in a quantitative sense but rather divided into low, moderate or high responders. The latter point becomes obvious as larger numbers of apparently healthy animals are analysed for lymphocyte blastogenesis; while those animals with negligible or very high stimulations tend to cluster, the majority of animals present as a continuum of responses ranging from low (10,000-20,000 CPM) to high (80,000-100,000 CPM) stimulations. The significance between animals on the low versus high end is unknown.

Based upon the above-mentioned problems associated with interpretation of blastogenesis relative to identification of immune system function/dysfunction, additional reagents and assays will be required. As described above, the adaptive immune system has been classically described as having two arms, cellular and humoral. Generation of a balanced immune response ensues following processing and presentation of the antigen by MHC class II-bearing cells of myeloid and lymphoid origin to a subpopulation of T lymphocytes that express the differentiation antigen, CD4. These CD4+T lymphocytes, T helper cells, play a pivotal role in facilitating the differentiation and expansion of antigen-specific B lymphocytes and CD8+ cytotoxic T lymphocytes. When considering establishment of techniques for measurement of marine mammal immune system function/dysfunction it is most logical to follow the advancements in human medicine. The extensive development of reagents and techniques that are currently providing advanced capabilities in assessing immune system status in humans, and many domestic and laboratory animal species, is lacking in marine mammals. Phenotypic and functional characterisation of an animals immune system has been dramatically enhanced in many species using panels of reagents including monoclonal antibodies specific for leukocyte differentiation and activation antigens and cytokine probes (monoclonal antibodies and/or cDNA probes to detect intracellular cytokine mRNA). Unfortunately, the majority of monoclonal antibodies, and many of the cDNA probes, are species-specific, requiring reagent development for individual marine mammal species. Development of monoclonal antibodies specific for cell-surface differentiation antigens, adhesion molecules and activation antigens is critical to understanding the immune systems of marine mammals and identifying acute to chronic perturbations in the system. To date, there is a complete absence of published reports describing such reagents for any marine mammal species other than monoclonal antibodies specific for harbour seal immunoglobulin (King *et al.*, 1993a) and antibodies specific for

human MHC class II proteins that cross-react with bottlenose dolphin MHC proteins (Romano *et al.*, 1992). While polyclonal antibody preparations have been developed that recognise surface Ig on B lymphocytes from a number of marine mammal species, antibodies specific for T cells and subsets thereof are lacking. From the authors' perspective, antibodies specific for CD3, a complex of invariant proteins associated with the T lymphocyte antigen receptor (TCR), could be considered a reagent in critical need relative to identification of immunocompromised marine mammals. Many of the CD3-specific monoclonal antibodies available for other species are capable of inducing T cell activation and proliferation in the absence of exogenous mitogens or interleukin(s). Not only can these reagents be used to enumerate circulating T lymphocytes by analytical flow cytometry, but can also activate T lymphocytes in a mechanism similar to that induced by antigen via the TCR. Such *in vitro* T cell activation is far more physiologically relevant than the polyclonal activation of lymphocytes via use of mitogens, LPS or phorbol esters. Thus, when attempting to assess T lymphocyte dysfunction, results will probably differ substantially between TCR- and mitogen-induced signal transduction and activation; we would speculate that activation induced via engagement of the TCR will identify immune suppression that would not be obvious following other non-physiologic activation mechanisms.

While lymphocyte blastogenesis, or lymphocyte proliferation, has classically been considered a measure of immune system function, advances in immunology have established that activation of lymphocyte subsets can result in production of cytokines (see below) with potent immunoregulatory capabilities; this attribute does not necessarily require cell proliferation. Thus, an additional assay gaining popularity for identifying immune system function measures the ability of lymphocytes to activate by identification of the expression of cell-surface activation antigens such as the IL-2 receptor (IL-2R). Such assays typically utilise analytical flow cytometry for enumeration of in vitro-activated lymphocytes (predominantly T cells) with fluorochrome-conjugated recombinant IL-2 or monoclonal antibodies specific for IL-2R (CD25). Not only can such analysis provide the absolute number of lymphocytes that were activated by a given stimulus but can also be used in two-colour analysis for identification of the lymphocyte subsets (e.g., CD4+ or CD8+) that have been activated. The species cross-reactivity exhibited by human recombinant IL-2 has permitted application of this technique to assessment of T cell function in a variety of marine mammals (dolphins, killer whales, sea otters and harbour seals). The commercial availability of fluorochrome-conjugated human IL-2 has made this flow cytometry technique possible. Upon development of monoclonal antibodies specific for lymphocyte differentiation antigens of marine mammal species, ability of multiple subsets to activate following an appropriate stimulus will provide an invaluable tool for assessing subtle levels of immune system dysfunction.

In addition to the physical interaction between antigen-presenting cells, T lymphocytes and B lymphocytes, an array of small proteins (cell-surface and/or secreted), cytokines, are pivotal in development and control of the immune response. Cytokines may exhibit autocrine (act upon the cell producing the cytokine), paracrine (act upon a nearby cell) and/or hormonal (act upon cells at a distant site) types of effects on receptor-bearing cells; the ability of a cell to express specific receptors plays a large role in controlling cytokine effects. A single cytokine can be produced by multiple cell types and induce different effects on different cell types. Furthermore, different cytokines can act in synergism or antagonism on a given cell. The development of reagents (monoclonal antibodies; generally species-specific) for identifying cytokines, transcription of cytokine genes and cytokine receptors has been extensive in human and marine species. Such analysis has provided a new dimension to understanding immune system function and dysfunction. Cytokine-dependent or responsive cell lines have been classically used to measure levels of multiple cytokines

with some being cross-species responsive. Harbour seal interleukin 6 (IL-6) has been identified in the plasma of animals with indications of systemic infection using such a cross-species bio-assay (King *et al.*, 1993b). IL-6 is a pleiotropic interleukin and becomes detectable in peripheral blood during an inflammatory response. Antigen-trapping enzyme-linked immunosorbent assays (ELISA) are beginning to find routine use in human medicine for identification of circulating levels of cytokines with hormonal type effects that are produced during an inflammatory response (tumour necrosis factor, IL-1 and IL-6) and thus have diagnostic value. An alternative approach currently being used for research purposes is identification of cytokines produced by individual cells via identification of their respective cytoplasmic mRNA. Many of the cytokine genes have sufficient cross-species homology to develop oligonucleotide primers for use in establishing polymerase chain reaction (PCR)-based identification of mRNA; this attribute is facilitating development of such probes in marine mammals. Such capabilities should prove valuable in the functional subdivision of CD4+T (T helper, TH) lymphocytes into those that facilitate inflammatory responses (TH1) versus those that facilitate Ig production (TH2); an evaluation of perturbations in such T cell subpopulations should prove very useful in the identification of subtle immune system perturbations.

In summary, advances are being made in improving our understanding of the immune systems of marine mammal species and such progress has the potential to accelerate in a manner analogous to that currently being experienced for human and marine systems. Establishment of the immune system as a sensitive monitor of the health of marine mammals will ultimately hinge upon acquisition of the above-mentioned reagents, techniques and application to normal healthy animals to establish baseline values.

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