Decreased Lymphocyte Responses in Free-ranging Bottlenose Dolphins (Tursiops truncatus) Are Associated with Increased Concentrations of PCBs and DDT in Peripheral Blood

Garet P. Lahvis,1 Randall S. Wells,2 Douglas W. Kuehl,3 Jennifer L. Stewart,4 Howard L. Rhinehart,5 and Charles S. Via6

1Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland; 2Chicago Zoological Society, Sarasota, Florida; 3U.S. Environmental Protection Agency, Environmental Research Laboratory, Duluth, Minnesota; 4Johns Hopkins School of Hygiene and Public Health, Baltimore, Maryland; 5Dolphin Biology Research Institute, Sarasota, Florida; 6University of Maryland School of Medicine and Research Service, Baltimore Veterans Administration Medical Center, Baltimore, Maryland

Since 1987, large-scale mortalities of dolphins have been reported along the Atlantic coast of North America, in the Gulf of Mexico, and in the Mediterranean Sea. Autopsied bottlenose dolphins, Tursiops truncatus, which were collected from the large-scale mortality along the Atlantic coast in 1987 to 1988, exhibited opportunistic infections indicative of immune dysfunction. Further, these animals had high levels of chlorinated hydrocarbons, such as PCBs and DDT, that can suppress immune functions. The purpose of this study was to determine whether there is a relationship between chemical contaminant exposure and immune response in free-ranging dolphins. In June of 1991, peripheral blood was obtained from members of a bottlenose dolphin population that resides along the west coast of Florida. Peripheral blood lymphocyte responses to Concanavalin A (Con A) and phytohemagglutinin (PHA) were determined in vitro and compared by regression analysis with contaminant concentrations in whole blood from a small subset of these animals (n = 5). These data indicate that a reduced immune response in these bottlenose dolphins was correlated with increasing whole blood concentrations of several contaminants. Specifically, inverse correlations were found between Con A-induced lymphocyte proliferation and tetrachlorinated to octachlorinated biphenyls (r² values ranged from 0.70 to 0.87). Con A-induced lymphocyte responses also correlated inversely with p,p' DDT (r² values of 0.73 and 0.79); o,p' - DDE (r² values of 0.93 and 0.96); and p,p' - DDE (r² values of 0.73 and 0.81).

— Environ Health Perspect 103(Suppl 4):67–72 (1995)

Key words: dolphin, Tursiops truncatus, marine, strandings, immune response, lymphocyte, contaminant, polychlorinated hydrocarbons, PCB (polychlorinated biphenyl), DDT (1,1,1-trichloro-2,2-bis(p-chlorophenylethyl)ethane)

Introduction

Since 1987, there have been several largescale dolphin mortalities. Between the midsummer of 1987 and the spring of 1988, over 740 bottlenose dolphins (Tursiops truncatus) were stranded along the Atlantic coast of North America (1). This single epizootic may have depleted the coastal migratory stock by as much as 53%, and population recovery may require as many as 100 years to return to pre-1987 population levels (1). In 1990 and again in 1992, there were two additional incidents of high or unusual mortality of bottlenose dolphins in the Gulf of Mexico (2). There has also been extensive mortality among striped dolphins (Stenella coeruleoalba) in the Mediterranean Sea (3–6). These incidents of high mortality have initiated scientific studies to determine their cause(s).

There are several commonalities among these large-scale mortalities. Geraci (7) described Atlantic coast stranded dolphins with epidermal lesions, possibly due to viral infection; dermal lesions presumed to be caused by bacteria, fungi or protozoans; and septicemia, a systemic bacterial infection. The bacteria identified included Edwardsiella spp., Streptococcus spp., Vibrio spp., Pseudomonas spp., Klebsiella spp., Acinetobacter spp., Bacillus spp., and Staphylococcus spp., with Vibrio spp. representing 52% of the total isolates. Bacteria were isolated from the liver, spleen, lung, lymph nodes, blood, urine, subcutaneous, abdominal fluid, kidney, and brain. Similar bacterial isolates were obtained from other stranded cetaceans along the Atlantic and Gulf coasts (8). Geraci concluded that these infections indicated that stranded dolphins were immunologically suppressed and were therefore less capable of surviving infectious diseases. Similarly, there was

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evidence of immunosuppression among the members of the striped dolphin population that were stranded along the coasts of the Mediterranean Sea (5). Lesions attributable to opportunistic fungi and bacteria in the oral cavity, brain, and lungs, and opportunistic parasitic infection of the lymph nodes, lungs, and brain were also reported in these animals (5). Immunosuppression was also inferred from studies of stranded beluga whales (Delphinapterus leucas) of the St. Lawrence Estuary (9).

One of the initial hypotheses to explain the cause of the 1987 to 1988 large-scale mortality of bottlenose dolphins was exposure to a natural algal neurotoxin, brevetoxin, that is produced by the marine dinoflagellate Pyrodiscus brevis. It is known that a large algal bloom, or red tide, of this species occurred during the summer of 1987 (7,10). It was therefore suggested that migrating dolphins ingested brevetoxin-contaminated fish and that neurotoxicity initiated a suppression of the immune system that resulted in opportunistic bacterial infection. In support of this hypothesis, it was found that 5 of 17 animals sampled from the mid-Atlantic coast die-off contained detectable concentrations of brevetoxin. However, it is equally important to note that brevetoxins could not be identified in the remaining nine dead dolphins. An independent study of the available data concluded that evidence for this hypothesis was circumstantial and that other explanations were also possible (10). Further, there does not appear to be evidence supporting a role for brevetoxins in the large-scale mortalities in the Gulf of Mexico or in the Mediterranean Sea.

A second hypothesis to explain the deaths of Atlantic bottlenose dolphins is that immune suppression occurred as a result of morbillivirus infection. Marine mammals from mortality events have been found with morbillivirus infections and rotaviruses, such as morbillivirus, can be immunosuppressive (11). Two common porpoises (Phocoena phocoena), for example, that were found dead on the coast of Northern Ireland were infected with morbillivirus (12). Morbillivirus infection was also identified in striped dolphins (Stenella coerulea alba) collected from the Mediterranean Sea in 1990. Among necropsied striped dolphins, the morbillivirus antigen was closely associated with microscopic lesions and was found in lymph nodes where there was also extensive lymphoid cell depletion (5). Recently, morbilliviral antigen was also identified in preserved dolphin samples from the 1987 to 1988 mortality event (13) and from a stranded bottlenose dolphin in the Gulf of Mexico (14).

However, although it is clear that morbillivirus infection can result in immunosuppression, the question remains: why were the dolphins susceptible to the morbillivirus infection initially? In the Mediterranean mortality event, morbillivirus infection was probably not the initial cause of the large-scale mortality because it rapidly invades lymph nodes and causes death within weeks (3); yet the increased prevalence of ectoparasites in the diseased animals (relative to healthy animals studied earlier) indicated that the immunosuppression may have begun a few months before the beginning of the epizootic (3,4). Thus the increased prevalence of parasites indicated that the dolphins may have been immunologically suppressed long before the morbillivirus infection. However, morbillivirus infection may have caused enhanced impairment of immune system function during later stages of bacterial, viral, and parasitic infection in each mortality event.

A third hypothesis was that chronic exposure to immunosuppressive pollutants, such as polychlorinated biphenyls (PCBs) and 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (p,p'-DDE), could have facilitated the development of viral, bacterial, and parasitic infections. Bottlenose dolphins stranded along the mid-Atlantic coast exhibited PCB and 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (p,p'-DDE), a metabolite of p,p'-DDE, that could facilitate the development of viral, bacterial, and parasitic infections. Bottlenose dolphins stranded along the mid-Atlantic coast exhibited PCB and 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (p,p'-DDE), a metabolite of p,p'-DDE, that could facilitate the development of viral, bacterial, and parasitic infections. Bottlenose dolphins stranded along the mid-Atlantic coast exhibited PCB and 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (p,p'-DDE), a metabolite of p,p'-DDE, that could facilitate the development of viral, bacterial, and parasitic infections. Bottlenose dolphins stranded along the mid-Atlantic coast exhibited PCB and 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (p,p'-DDE), a metabolite of p,p'-DDE, that could facilitate the development of viral, bacterial, and parasitic infections. Bottlenose dolphins stranded along the mid-Atlantic coast exhibited PCB and 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (p,p'-DDE), a metabolite of p,p'-DDE, that could facilitate the development of viral, bacterial, and parasitic infections.
Lymphocyte Responses and Contaminants in Dolphins

After centrifugation (200g for 20 min), cells were harvested from the interface and resuspended in culture medium containing RPMI 1640, 10% fetal calf serum, 100 U penicillin/streptomycin, 1% glutamine, 1% nonessential amino acids, 1% pyruvate, and 0.05% 2-mercaptoethanol. Cells were cultured at 37°C in 6 to 8% CO₂ from 1 to 8 days in 96-well flat-bottom microtiter tissue culture plates (4x10³ cells/well) and were either unstimulated or stimulated with the mitogens Concanavalin A (Con A) or phytohemagglutinin (PHA) at the concentrations indicated. Following an overnight pulse of 9 hr with ³H-thymidine (1.0 µCi), cells were harvested and thymidine uptake was measured by liquid-scintillation counting. Results were expressed as mean counts per min (cpm) for three replicate wells. Standard errors were typically less than 15% and have been omitted. We have observed that in vitro lymphocyte responses are altered by storage of samples for greater than 48 hr prior to analysis and by changes in fetal calf serum lots (unpublished observations). For these reasons, lymphocyte separation was initiated within approximately 24 hr of obtaining the sample, and the same batch of fetal calf serum was used for all tests. Because the time to peak proliferation can vary among dolphins, proliferative responses were assayed daily for between 1 and 8 days of culture, and the maximal proliferation value for each individual dolphin in response to a given mitogen concentration was determined.

Chemical Analysis

Samples were analyzed for polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans, PCBs, pesticides, and other chlorinated compounds using established protocols (26–29). Briefly, each sample of peripheral blood (10 ml) was treated with methanol to lyse cells, dried with anhydrous sodium sulfate, and fortified with internal standards. Samples were soxhlet extracted overnight with 400 ml of solvent (1:1 v/v hexane/methylene chloride) and reduced in volume with a Kuderna-Danish apparatus to 1 ml. Analytes were isolated from the extracted lipid by chromatography on silica gel and carbon on silica gel. Polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and coplanar PCBs were quantified using high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS) (Finnigan-MAT model 8230). Pesticides and total PCBs were quantified using HRGC/low resolution mass spectrometry (LRMS) (Finnigan-MAT model 4500). High and low resolution GCs were fitted with temperature programmed 30m DB-5 fused silica columns. Quantification and quality assurance/quality control were conducted as previously described (28,29).

Results

Lymphocytes were isolated from peripheral blood of 15 dolphins and cultured in the presence of Con A or PHA. In this study, lymphocyte proliferation was consistently strongest when concentrations of Con A were 0.5 and 0.13 µg/ml and concentrations of PHA were 2.0 and 0.5 µg/ml (data not shown). Table 1 shows the peak proliferative responses of all 15 male dolphins. Dolphin FB 156 exhibited the lowest responses to Con A at both concentrations and exhibited nearly the lowest responses to PHA. Similarly, dolphin FB 24 showed the highest response to both mitogens.

Based upon these data, five males were selected for chemical residue analysis. High cost precluded residue analysis for all male dolphins, so 5 of the 15 dolphins were selected for contaminant analysis. Individual samples which were either relatively low or high with regards to mitogen stimulation were analyzed for pollutant residues. PCBs: p,p′-DDT; o,p′-DDE; and p,p′-DDE were found in most individuals (Table 2). Hexachlorinated PCBs represented the greatest fraction of total PCBs, though pentachlorinated and heptachlorinated PCBs also contributed substantially to total peripheral blood PCB levels. p,p′-DDE levels were more than 10-fold higher than p,p′-DDT levels, and at least two orders of magnitude greater than blood levels of o,p′-DDE levels.

Linear regression analysis was used to determine the relationship between peripheral blood contaminant concentration and lymphoproliferative responses to mitogens. Figures 1A and B illustrate respectively the inverse correlation between lymphocyte proliferative responses to Con A and pentachlorinated (r² = 0.87) and hexachlorinated (r² = 0.84) PCB levels. Figures 2A and B

Table 1. Maximal mitogen-induced proliferative responses for 15 male dolphins.²

| Dolphin | ID | Con A, 0.5 µg/ml | Con A, 0.13 µg/ml | PHA, 2.0 µg/ml | PHA, 0.5 µg/ml |
|---------|----|-----------------|------------------|----------------|---------------|
| FB 20   | 35115 | 60332           | 24864            | 32950           |
| FB 24   | 100088 | 86390           | 94512            | 74819           |
| FB 26   | 36764 | 63240           | 32233            | 32052           |
| FB 32   | 55130 | 75859           | 54646            | 73592           |
| FB 46   | 77178 | 75169           | 65990            | 62508           |
| FB 49   | 33830 | 38473           | 37305            | 24277           |
| FB 98   | 47173 | 90080           | 59955            | 41553           |
| FB 104  | 43031 | 47088           | 22383            | 43750           |
| FB 108  | 54044 | 94666           | 52767            | 33546           |
| FB 132  | 29294 | 47590           | 21575            | 42633           |
| FB 154  | 62142 | 49129           | 48561            | 38482           |
| FB 156  | 33110 | 33505           | 28030            | 24735           |
| FB 162  | 63164 | 34371           | 42066            | 43433           |
| FB 164  | 65092 | 70110           | 41140            | 49648           |
| FB 168  | 71494 | 50999           | 55390            | 66442           |

*In vitro proliferation to Con A or PHA was performed as described in "Materials and Methods." Values represent peak proliferative response (in counts per minute) for each mitogen. *Dolphins were chosen for further analysis of contaminant concentrations.

Table 2. Analytical chemistry, mitogen-induced lymphoproliferative responses, and ages of five male dolphins.²

| Parameter | FB46 | FB48 | FB156 | FB164 | FB168 |
|-----------|------|------|-------|-------|-------|
| Proliferation |     |      |       |       |       |
| Con A, 0.5 µg/ml | 77178 | 33300 | 33110 | 65092 | 71494 |
| Con A, 1.3 µg/ml | 75169 | 38473 | 33505 | 70110 | 50999 |
| PHA, 2.0 µg/ml | 65990 | 37306 | 28030 | 41140 | 55390 |
| PHA, 0.5 µg/ml | 62508 | 24735 | 49648 | 66442 |       |
| Analytical chemistry |     |      |       |       |       |
| Trichloro PCBs | 2.6 | 6.5 | 8.8 | ND | 2.8 |
| Tetrachloro PCBs | 5.8 | 15.1 | 17.6 | 1.5 | 9.1 |
| Pentachloro PCBs | 13.5 | 44.0 | 53.4 | 3.7 | 21.1 |
| Hexachloro PCBs | 107.0 | 304.0 | 322.1 | 12.5 | 150.9 |
| Heptachloro PCBs | 59.2 | 293.0 | 260.1 | 6.6 | 88.1 |
| Octachloro PCBs | 16.7 | 81.4 | 59.0 | 2.0 | 22.5 |
| Nonachloro PCBs | 2.2 | 7.0 | 4.2 | ND | 2.3 |
| Decachloro PCBs | 0.1 | 1.0 | 0.7 | ND | 0.4 |
| p,p′-DDT | 0.9 | 22.0 | 2.3 | ND | 1.3 |
| o,p′-DDE | 108.1 | 536.3 | 396.5 | 12.7 | 130.5 |

Approximate age, years | 13 | 32 | 21 | 3 | 9 |

ND, the analyte was not detected. *Contaminant concentrations determined for whole blood as determined in "Materials and Methods" and expressed in nanogram per gram. For convenience, mitogen-induced proliferative responses from Table 1 are also shown.
show a similar inverse correlation between lymphocyte responses to Con A and peripheral blood p,p'-DDT ($r^2 = 0.79$) and p,p'-DDE concentrations ($r^2 = 0.81$). In one of the five animals, p,p'-DDT was not detected. For linear regression analysis, we have arbitrarily assigned this individual a value for p,p'-DDT of one-half of the detection limit.

Proliferative responses correlated inversely with contaminant levels of several PCB congeners, p,p'-DDT; o,p'-DDE; and p,p'-DDE. $r^2$ values for the correlation between Con A- and PHA-induced lymphoproliferation, and the concentration of DDT and DDT metabolites and total trichlorobiphenyl to decachlorobiphenyl congeners are presented in Table 3. Inverse correlations ($r^2$ values greater than 0.75) were often observed between total trichlorobiphenyl to heptachlorobiphenyl congeners and Con A-induced lymphoproliferative responses. Inverse correlations were also observed between DDT and DDT metabolites and Con A-induced lymphocyte responses. Weak correlations, or lack of correlations ($r^2$ values less than 0.60), were often exhibited between blood contaminant levels and PHA-induced lymphocyte responses and may be accounted for by the single depressed response of FB164 to PHA.

**Discussion**

In an effort to test the hypothesis that polyhalogenated environmental chemical contaminants suppress the immune system of dolphins, thereby causing susceptibility to infection and mortality, we have studied contaminant concentrations in blood and lymphocyte proliferation responses to mitogens for a small set of male bottlenose dolphins. These data indicate that in bottlenose dolphins a reduced *in vitro* immune response is associated with increasing levels of PCBs and DDT in peripheral blood. The small sample size in this study ($n=5$) and the lack of control (uncontaminated) dolphins from which we can determine the normal range of immune responses, precludes drawing extensive conclusions. However, these data are consistent with the results of other studies which show that PCBs and DDT can suppress immune responses.

Abundant evidence generated from controlled animal studies indicates that PCBs and DDT suppress immune responses. PCB-induced immunosuppression has been documented in mice (29-32), rats (33), guinea pigs (34), ducks (35), monkeys (36-38), and possibly in humans (39). Exposure to PCBs can result in reduced relative spleen (31,32,40) and thymus (40) size, decreased T cell-dependent antibody-forming cell (AFC) formation (31,32), lower T cell-dependent antibody titer (32,36,39), depressed cytotoxic T lymphocyte (CTL) response (40), and reduced delayed-type hypersensitivity (39). Further, PCB exposure can reduce lymphocyte proliferation responses to phytohemagglutinin (33,38) and can alter natural killer (NK) cell activity (33). Experimental studies with rodents have shown that DDT exposure can result in decreased spleen/body ratio (41-43) and decreased lymphocyte response to lipopolysaccharide (LPS) (44). Further, T cell-dependent (42) and T cell-independent (42,44,45) B cell responses can be depressed by exposure to DDT. Thus, it is biologically plausible that immune suppression of bottlenose dolphins can result from PCB and/or DDT exposure.

Exposure to PCBs can render animals more susceptible to viral and bacterial infection. Mallard ducklings (*Anas platyrhynchos*) that were exposed to PCBs exhibited higher mortality than unexposed ducklings after challenge with duck hepatitis (35). Similarly, PCB exposure can increase mouse susceptibility to malaria (*Plasmodium berghei*) infection (27), to *Salmonella* spp. (29,36), and to both ectromelia virus and Herpes simplex virus infection (30). Again, this suggests the possibility that the dolphins that were stranded and showed mortality-associated infectious diseases may have become more susceptible to a virus, such as morbillivirus, as a result of exposure to these compounds.

Environmentally relevant concentrations of PCBs have also been shown to impair immunity of harbor seals (*Phoca vitulina*), which have experienced large-scale mortality events (due, in part, to the morbillivirus-related distemper virus) in Europe since 1988. A recent study shows that harbor seals exhibited depressed
immune responses if fed contaminated fish from the Baltic Sea rather than cleaner fish from the Atlantic Ocean (46).

While the data presented here are preliminary, they are consistent with other studies that have found high levels of pollutants in dolphins, with laboratory findings demonstrating the effects of PCBs and DDT on the immune system, and with data showing that environmentally relevant levels of PCBs in fish can cause immunosuppression in other marine mammals. Future work must be conducted to determine whether a larger sample size can also support the correlations found in this study and, if so, if specific pollutants are responsible for the decreased lymphocyte proliferation response.

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### Table 3. r² values of linear regressions for mitogen-induced lymphocyte proliferation versus whole blood pollutant levels.

| Chemical analyte | Con A, 0.5 µg/ml | Con A, 0.13 µg/ml | PHA, 0.2 µg/ml | PHA, 0.5 µg/ml |
|------------------|------------------|------------------|----------------|----------------|
| Trichloro PCBs   | 0.72             | 0.77             | 0.40           | 0.61           |
| Tetrachloro PCBs  | 0.70             | 0.87             | 0.36           | 0.54           |
| Pentachloro PCBs  | 0.79             | 0.87             | 0.46           | 0.65           |
| Hexachloro PCBs   | 0.73             | 0.84             | 0.34           | 0.59           |
| Heptachloro PCBs  | 0.84             | 0.74             | 0.42           | 0.73           |
| Octachloro PCBs   | 0.79             | 0.74             | 0.34           | 0.68           |
| Nonachloro PCBs   | 0.58             | 0.58             | 0.16           | 0.51           |
| Decachloro PCBs   | 0.59             | 0.63             | 0.16           | 0.48           |
| a.p.-DDT          | 0.79             | 0.73             | 0.34           | 0.70           |
| a.p.-DDE          | 0.96             | 0.93             | 0.96           | 0.87           |
| p.p.-DDE          | 0.81             | 0.73             | 0.36           | 0.72           |

*The r² values were generated from linear regression analyses. We opted for a linear regression for statistical simplicity and do not suggest that this defines the nature of a dose–response relationship.*
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