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Hematological, Biochemical, and Immunological Findings in Atlantic Bottlenose Dolphins (*Tursiops truncatus*) with Orogenital Papillomas

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Abstract

The first cases of orogenital sessile papillomas associated with a novel gammaherpesvirus and papillomavirus were recently reported in free-ranging Atlantic bottlenose dolphins (*Tursiops truncatus*). The tumors appear to be sexually transmitted and are now occurring in epidemic proportions in some coastal areas. This study describes the hematological, biochemical, and immunological findings in free-ranging bottlenose dolphins with orogenital papillomas from the coastal waters of South Carolina and Florida. Blood samples were obtained from 22 dolphins with papillomas and 86 healthy dolphins. Few statistically significant differences were found for hematological and serum chemistry variables. Serum iron was significantly lower and serum bicarbonate significantly higher in dolphins with orogenital papillomas compared with healthy dolphins. However, dolphins with tumors had multiple abnormalities in serum proteins and immunologic parameters. Serum protein electrophoresis results demonstrated significantly elevated levels of total globulin, total alpha globulin, and alpha-2 globulin in dolphins with orogenital papillomas. Gamma globulins were also elevated in dolphins with orogenital papillomas although not significantly. Innate immunity was up-regulated in dolphins with tumors. Granulocytic and monocytic phagocytosis and superoxide respiratory bursts were significantly higher in dolphins with orogenital tumors compared with healthy dolphins. Adaptive immunity appeared to be relatively intact with an up-regulated humoral immune response; statistically significant increases were found in B-lymphocyte proliferation and antibody titers to the common marine microorganisms *Escherichia coli*, *Erysipelothrix rhusiopathiae*, *Mycobacterium marinum*, *Vibrio cholerae*, *V. carchariae*, *V. vulnificus*, and *V. parahemolyticus*. The only adaptive immune system variable significantly lower in dolphins with tumors was the percentage of lymphocytes expressing MHC class II molecules, suggesting possible impaired or down-regulated expression as has been noted in humans with viral infections. The clinically relevant results indicate that dolphins with orogenital papillomas demonstrate hypoferremia, hyperglobulinemia, and hyperalphaglobulinemia likely associated with an acute-phase inflammatory response and up-regulated innate and humoral immunity, all possible responses to the tumors and/or the viruses associated with the tumors. Also, dolphins with orogenital papillomas may have enhanced innate and humoral adaptive immunity because of increased exposure to other directly transmitted pathogens.

Key Words: Atlantic bottlenose dolphin, *Tursiops truncatus*, orogenital papilloma, emerging disease, hematology, serum analytes, immunology

Introduction

The free-ranging Atlantic bottlenose dolphin (*Tursiops truncatus*) Health and Risk Assessment

(HERA) project was initiated as a multidisciplinary, integrated collaborative effort in 2003 to assess individual and population health in two southeast coastal regions: Charleston (CHS), South Carolina (SC), and the Indian River Lagoon (IRL), Florida (Bossart et al., 2006). In part, the goals of the project were to develop standardized tools for health and risk assessment and to explore associations between health status and environmental stressors. As an apex predator, bottlenose dolphins serve as a sentinel species for monitoring the health of the environment and may provide valuable information to evaluate the relationship between exposure to biological and chemical agents and adverse health effects for these populations (Bossart, 2006).

As part of the HERA project, the first cases of orogenital papillomas were reported in free-ranging bottlenose dolphins from Atlantic coastal waters. It was postulated that the tumors had a similar etiopathogenesis and orogenital route of transmission and might represent one or more emerging diseases of viral etiology (Bossart et al., 2005; Rehtanz et al., 2006). A goal from the initial study was to define various clinicopathologic factors of dolphins with orogenital papillomas that would help elucidate associated health issues related to this novel neoplastic disease. The purpose of this report is to evaluate the hematological, biochemical, and immunological findings in dolphins with orogenital papillomas and to describe the rapid emergence of this disease.

Materials and Methods

Dolphin Sampling

One hundred and seventy-one bottlenose dolphins were sampled in a cross-sectional study during the summers of 2003, 2004, and 2005 as part of a comprehensive dolphin health assessment in CHS and the IRL (Bossart et al., 2006; Reif et al., 2008). Eighty-six (50.3%) dolphins were considered healthy (i.e., to the best of the authors' knowledge they were free of disease) based on a veterinary review of data from physical examination; ultrasound examination; hematology; serum chemistry; gastric, blowhole, and fecal cytology; urinalysis; and the results of microbiologic testing of samples obtained from the blowhole and rectum as reviewed previously (Bossart et al., 2006; Fair et al., 2006; Goldstein et al., 2006). Sixteen (9.4%) dolphins had grossly and histologically confirmed orogenital sessile papillomas on initial capture as described previously in free-ranging and captive dolphins (Bossart et al., 2005). Additionally, 15 dolphins were captured more than once. Thirteen were captured in two yearly samples, and two dolphins were captured in each of the three sampling years, one at each site. Six of the 15 (40.0%) dolphins developed an orogenital papilloma between their first and second captures (2 of 7 from CHS [28.5%] and 4 of 8 from the IRL [50.0%]).

Incisional biopsies were aseptically obtained from oral and genital lesions following local anesthesia by infiltration of 2% lidocaine hydrochloride. Tissues were placed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for examination by light microscopy. Age was estimated by examination of postnatal dentine layers in an extracted tooth (Hohn et al., 1989). For this study, adults were defined as dolphins 6 y of age or older as described previously (Bossart et al., 2005).

Blood Collection

Blood samples were drawn from the periarterial venous rete in the flukes, generally within the first 15 min of capture, with a 19-gauge, 1.9-cm butterfly catheter (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) (Goldstein et al., 2006). Serum was collected in 10-ml separator vacutainer tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA), placed in a cooler for between 20 and 40 min, and centrifuged for 15 min at 1,200 rpm. Samples for hematology and immunology were collected in a vacutainer tube with ethylene diamine tetraacetic acid (EDTA) or sodium heparin as an anticoagulant, respectively (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Samples for hematology, serum chemistry, and serum protein electrophoresis analyses were stored in an insulated cooler and shipped overnight to the Cornell University Veterinary Diagnostic Laboratory in Ithaca, New York, USA. Immunological samples (heparinized whole blood or serum as required) were stored in an insulated cooler and shipped overnight to Mystic Aquarium & Institute for Exploration, Clemson University, and the Medical University of South Carolina for analysis.

Hematology, Serum Chemistry, and Serum Protein Electrophoresis

For the complete blood count, relative leukocyte determinations were performed by microscopic examination of modified Wright-stained blood smears (Bayer Healthcare, Tarrytown, NY, USA). A microhematocrit tube was centrifuged for 5 min at 11,700 rpm, and the manual hematocrit was interpreted by visual inspection against a standard calibration. Automated hemoglobin, red blood cell count, mean corpuscular platelet volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), white blood cell count (WBC), and total platelets were determined by

an automated analyzer (Bayer ADVIA 120, Bayer Diagnostics, Tarrytown, NY, USA). The concentrations of serum chemistry analytes were determined with an automated analyzer (Hitachi 917, Roche, Indianapolis, IN, USA) and included analyses for sodium, potassium, chloride, bicarbonate, anion gap, blood urea nitrogen (BUN), creatinine, uric acid, calcium, phosphorus, magnesium, glucose, total direct and indirect bilirubin, cholesterol, triglycerides, iron, and fibrinogen. Enzyme activity was determined for alanine aminotransferase (ALT), alkaline phosphatase, aspartate aminotransferase (AST), creatinine phosphokinase (CPK), gamma-glutamyltransferase (GGT), lactate dehydrogenase (LDH), amylase, and lipase. Fibrinogen concentration was determined by the method of Schalm using heat precipitation. Serum protein electrophoresis was done on an automated analyzer (Rapid Electrophoresis, Helena Laboratories, Beaumont, TX, USA) as previously described (Goldstein et al., 2006). Hemolyzed or lipemic samples were not included.

Immune Assays

Unless otherwise specified, all chemicals and mitogens used for immune assays were purchased from Sigma (St. Louis, MO, USA). Non-essential amino acids (NEAA; 10 mM 100X), sodium pyruvate (100 mM), and Hank's balanced salt solution (HBSS) were obtained from GIBCO Laboratories (Grand Island, NY, USA). RPMI-1640 medium (with L-glutamine and sodium bicarbonate), phosphate buffered saline (PBS; with or without Ca^{+2} and Mg), and penicillin/streptomycin (5,000 IU/mL, 5,000 µg/mL) were purchased from Cellgro (Mediatech, Herndon, VA, USA). The radioisotopes, sodium chromate (^{51}CR; specific activity 351 mCi/mg^{Cr}), and tritiated (^{3}H; specific activity 71 Ci/mmol) thymidine were acquired from ICN (Costa Mesa, CA, USA). The fetal bovine serum (FBS) was from Gemini Bio-Products (West Sacramento, CA, USA). Luma Plates™, Unifilters®, and Microscint 20™ were procured from Packard (Meriden, CT, USA). YAC-1 cells were purchased from ATCC (Manassas, VA, USA). Triton X, zap-o-globin, tissue culture plates, 2-mercaptoethanol, and disposables were purchased from Fisher Scientific (Atlanta, GA, USA). Propidium idodide was purchased from Invitrogen (Grand Island, NY, USA), and the Staphlococcus aureus was obtained from ATCC (Manassas, VA, USA). Antibodies were previously used in other published studies. Q5/13 is a monoclonal antibody that cross-reacts with dolphin class II molecules (Romano et al., 1992). CD2, CD19, and CD21 are cetacean-specific monoclonal antibodies that recognize T lymphocytes, and mature and immature B lymphocytes, respectively (De Guise et al., 2002). CD4, a cell surface marker on T lymphocytes, were labeled with a monoclonal antibody to human CD4 that cross-reacts with cetacean CD4 (De Guise et al., 1997) and a dolphin-specific CD4 antibody (Romano et al., 1999). The secondary antibody, fluorescein isothiocyanate conjugated affinity purified goat anti-mouse F(ab)'$_2$ IgG was commercially available through Beckman Coulter (Miami, FL, USA).

Leukocyte Isolation and Counting

For assessment of proliferation, NK-cell activity, superoxide production, and immunophenotyping, peripheral blood leukocytes (PBLs) were isolated by a slow spin technique within 36 h of blood collection as previously described (Keller et al., 2006) with minor modifications. Briefly, whole blood was centrifuged at 700 rpm (59 × g) in a swinging bucket rotor for 25 min at 8º C. PBLs were collected by gently swirling the buffy coat into the plasma and transferring the cells into a new tube. Following centrifugation at 1,500 rpm (377 × g) for 5 min, the plasma was removed and the cell pellet was gently resuspended in 1 mL of complete media (RPMI-1640, 10% fetal bovine serum, 50 IU penicillin, and 50 µg streptomycin). Numbers of nucleated PBLs were determined using a hemacytometer following red blood cell lysis with zap-o-globin or 0.17 M ammonium chloride. All counts were performed by one person to ensure consistency. Cells were then diluted as described for each endpoint.

Immunophenotyping

Lymphocyte subsets were labeled and analyzed according to methods described previously (Romano et al., 2004). Briefly, 1 × 106 cells/ml were labeled with 50 µL of monoclonal supernatant for 30 min at 4° C. Monoclonal supernatant of the myeloma cell line P2X63-AG8.653 was used as a negative control. The cells were washed three times with HBSS before incubation with fluorescein isothiocyanate conjugated affinity purified goat anti-mouse F(ab)'$_2$ IgG for 30 min at 4° C in the dark. Cells were then washed twice in PBS and resuspended in 500 µL of 1% paraformaldehyde for analysis by flow cytometry. Samples were analyzed on an LSR flow cytometer (BD Biosciences, San Jose, CA, USA). Forward/side scatterplots were obtained for each subject. Lymphocytes were gated based on their size and low degree of granularity. Ten thousand gated events were analyzed by histogram statistics.

Phagocytosis

As one measure of innate immunity and antigen-presenting capabilities bridging to adaptive immunity, the percent phagocytosis for phagocytic cell

types (i.e., granulocytes and monocytes) was determined using a modification of the technique described by Flaminio et al. (2002) and Lehmann et al. (2000). Briefly, cell counts on heparinized whole blood were performed as described above. One hundred microliters of cells were aliquoted into each 12 × 75 mm, 5-ml round bottom BD Falcon™ tube (BD Biosciences, San Jose, CA, USA) for a total of 12 tubes in triplicate. Ten microliters of 2.1×10^9 units/ml heat-killed *Staphlococcus aureus* previously labeled with 100 µg/ml of propidium iodide was added to each tube for a 25:1 bacteria:cell ratio. Control tubes contained cells with unlabelled bacteria or cells alone. Tubes were incubated in a shaking 37° C water bath (100 rpm) for 0 and 75 min (prior time course experiments indicate 75 min at the optimal time point). The experiment was stopped by adding 10 µl of 1 mM N-ethylmaleimide (NEM) to each tube. Tubes were immediately placed on ice. Red blood cells were lysed by the addition of 1 ml of lysis buffer (0.01 M Tris/0.001 M EDTA/0.17 M NH_4CL, pH = 7.4). After a 15-min incubation at room temperature, cells were washed 3x in 1 ml PBS. Following the last wash, cells were resuspended in 250 µl of 1% cold paraformaldehyde, pH 7.4. Cells were analyzed on an LSR flow cytometer (BD Biosciences, San Jose, CA, USA). Forward/side scatterplots were obtained for each subject. Monocytes and granulocytes were gated based on their size and degree of granularity. One hundred thousand gated events were analyzed by histogram statistics.

Lysozyme Activity

Lysozyme activity, another measure of innate immunity, was assessed using slight modifications of a standard turbidity assay (Demers & Bayne, 1997; Keller et al., 2006). A 1 mg/mL stock solution of hen egg lysozyme (HEL; Sigma, St. Louis, MO, USA) was prepared in 0.1 M phosphate buffer (pH 5.9), and aliquots were frozen until use. A solution of *Micrococcus lysodeikticus* was prepared fresh daily by dissolving 50 mg of the lyophilized cells in 100 mL, 0.1 M phosphate buffer (pH 5.9). HEL was serially diluted in phosphate buffer to produce a standard curve of 40, 20, 10, 5, 2.5, 1.25, 0.6, 0.3, and 0 µg/µL. Aliquots of each concentration (25 µL/well) were added to a 96-well plate in triplicate. For each sample, 25 µL of test plasma was added in quadruplicate to the plate. The solution of *M. lysodeikticus* (175 µL/well) was quickly added to three sample wells and to each of the standard wells. The fourth well containing plasma received 175 µL phosphate buffer and served as a blank. Plates were assessed for absorbance at 450 nm with a spectrophotometer (SpectraCount, Packard, Meriden, CT, USA) immediately (T0) and again after 5 min (T5). Absorbance unit (AU) values at T5 were subtracted from AU values at T0 to determine the change in absorbance. The AU value for the blank sample well was subtracted from the average of the triplicate sample wells to compensate for any hemolysis or color variation in the samples. The resultant AU value was converted to HEL concentration (µg per µl) via linear regression of the standard curve.

Mitogen-Induced Lymphocyte Proliferation (LP)

The LP response was measured in order to determine the ability of lymphocytes to undergo clonal expansion in response to mitogens using methods optimized previously (Peden-Adams & Romano, 2005). Briefly, isolated viable peripheral blood leukocytes (PBLs) were diluted to 1.0×10^6 cells/mL in supplemented RPMI-1640 medium (RPMI-1640, 10% fetal bovine serum, 1% nonessential amino acids, 1% sodium). One hundred µL aliquots of the resulting cell suspensions were dispensed into 96-well plates (1×10^5 cells/well) containing triplicate wells of 2.5 µg/mL concanavalin A (Con A; type IV-S; a T-cell mitogen), 120 µg/mL lipopolysaccharide (LPS; *E. coli* 055:B5; a B-cell mitogen), or supplemented RPMI-1640 (unstimulated wells). All mitogen concentrations are expressed in µg/mL of culture (final culture concentration; culture volume was 200 µL). Positive and negative controls were run for each animal. Plates were incubated for 96 h at 37° C and 5% CO_2. After 96 h, 0.5 µCi of tritiated thymidine in RPMI-1640 was added to each well in a volume of 100 µL. Sixteen hours later, cells were harvested onto Unifilter plates using a Packard Filtermate™ 96-well plate harvester (Packard, Meriden, CT, USA) and the plates were allowed to dry. Once dry, 25 µL of Microscint 20™ was added to each well, and the samples were analyzed using a Packard Top Count™-NXT scintillation counter (Packard, Meriden, CT, USA).

Natural Killer Cell Activity

Natural killer (NK) cells are important in innate immunity specifically with tumor surveillance and killing cells with intracellular infections. NK cell activity was assessed via an *in vitro* cytotoxicity assay using ^{51}Cr-labeled Yac-1 cells as described previously with slight modifications (Peden-Adams et al., 2007). To minimize radioactive waste, the procedure was adapted to 96-well plates that were read on a Packard Top Count scintillation counter. PBLs were adjusted to 1×10^7 nucleated cells/mL in complete medium (RPMI-1640, 10% fetal bovine serum, 50 IU penicillin, and 50 mg streptomycin). PBLs and Yac-1 cells were prepared, in triplicate, in ratios of 100:1, 50:1, and

25:1. Maximum release was determined by lysing ^{51}Cr-labeled Yac-1 cells with 0.1% Triton X in complete media. Spontaneous release was determined by incubating Yac-1 tumor cells only in complete media. After a 6-h incubation at 37° C and 5% CO_2, the plates were centrifuged (377g; 5 min), and 25 µL of supernate was then transferred to a 96-well plate containing solid scintillant (LumaPlate™). A mouse positive control was run with all samples to ensure test reliability. Plates were air dried overnight, and then counted for 5 min, after a 10-min dark delay, on the Packard Top Count™-NXT (Packard, Meriden, CT, USA).

Superoxide Production

Superoxide production, a measure of respiratory burst and thus innate immunity, was determined using PBLs by assessing nitroblue tetrazolium (NBT) conversion. Briefly, PBLs were diluted to 5 × 10^6 cells/mL in complete medium (RPMI-1640, 10% fetal bovine serum, 50 IU penicillin, and 50 µg streptomycin). One hundred µL aliquots of the resulting cell suspensions were dispensed into 96-well plates containing triplicate wells of 60 µL of calcium ionphore (CI; 6.5 µL/mL complete media solution made from a 1 mg/mL DMSO frozen stock), 12, 13-phorbol dibutyrate (PDB; 12.4 µL/mL complete media solution made from a 1 mg/mL DMSO frozen stock), or supplemented RPMI-1640 (unstimulated wells). To this, 140 µg NBT (10 mg NBT in 56.8 mL of HBSS with $CaCl_2$ [2mM]) was added to each well. Plates were incubated for 10 min at 37° C and 5% CO_2. Plates were then centrifuged at 1,500 rpm (377 × g) for 3 min and the supernate was removed. KOH (120 mL of 2N) and 140 mL DMSO were then added to each well. Solutions in each well were then pipetted up and down carefully to mix, and plates were assessed for absorbance at 620 nm with a spectrophotometer (SpectraCount; Packard, Meriden, CT, USA). Results were reported as the stimulation index (AU stimulated/AU unstimulated).

Antibody Titers Against Marine Bacteria

As an indication of a general humoral response to common marine bacteria, specific antibody titers against select marine bacteria were determined in an ELISA system as described previously (Beck & Rice, 2003). Cultures of the following bacteria were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in their recommended broths: *Escherichia coli*, *Erysipelothrix rhusiopathiae*, *Mycobacterium marinum*, *Vibrio cholerae*, *V. carchariae*, *V. vulnificus*, and *V. parahemolyticus*. Calculations of serum antibody titers from individual dolphins following ELISAs were then expressed in terms of antibody titers at a 1:200 serum dilution (Karsten & Rice, 2004).

Statistical Analyses

Statistical analyses were conducted in *SAS*, Version 9.1 (SAS Corp, Cary, NC, USA). The data were checked for normality by use of the Shapiro-Wilk statistic. Mean values (± SD) for each parameter for dolphins with orogenital neoplasia (n = 22) were compared with healthy dolphins (n = 86) by use of the nonparametric Wilcoxon rank-sum test because of the small number of observations in the diseased group and non-normal distributions for multiple parameters, with $p < 0.05$ as the level for statistical significance. Categorical data for prevalence and sex were compared with use of the Chi-square test.

Pre- and post-disease analysis of the six recaptured dolphins that developed an orogenital papilloma was restricted to parameters for which statistically significant differences were found between normal dolphins and those with orogenital papillomas in the main analysis. Statistical significance was determined by use of the nonparametric Wilcoxon rank sum test with computation of exact p values because of the small number of subjects. Analysis was limited to parameters with at least five observations for pre- and post-disease samples.

Results

The 171 individual dolphins captured contributed 188 evaluations, including recaptures. None of the 89 dolphins captured in 2003 had evidence of a papilloma. The first evidence of papillomas was found in 2004 when 10 of 61 captures from both sites (16.4%) showed evidence of an oral or genital papilloma. In 2005, the prevalence rose to 31.6% (12 of 38 captures). The prevalence of orogenital neoplasia was approximately twice as high in dolphins from the IRL (15/98, 15.3%) as in dolphins from CHS (7/90, 7.8%). However, the difference in prevalence was not statistically significant. The distributions of age and sex were compared between dolphins with orogenital papillomas and healthy dolphins. The mean ages for healthy dolphins and dolphins with orogenital papillomas were 10.7 and 8.1 y, respectively. Of the 22 dolphins with orogenital papillomas, 18 were adults and three were subadults supporting an adult predisposition for the tumors as previously reported (Bossart et al., 2005). Males were slightly over-represented among healthy dolphins (63.6% male) as well as among dolphins with orogenital papillomas (57.0% male). None of the differences in age and sex between healthy dolphins and those with orogenital papillomas were statistically significant.

Hematology and serum analyte results are summarized for healthy dolphins and dolphins with

orogenital papillomas in Table 1. Statistically significant changes were found for only two of these variables. Serum iron was significantly lower and serum bicarbonate significantly higher in dolphins with orogenital papillomas compared with healthy dolphins. No significant differences were found in the remaining tested hematological and serum biochemical analyte results, although borderline near-significant decreases were found for serum cholesterol and lipase ($p = 0.05$ for each).

Immunological data are summarized in Table 2. B-lymphocyte proliferation, granulocytic and

Table 1. Hematology and serum analyte results in healthy dolphins ($n = 86$) and dolphins with orogenital papillomas ($n = 22$)

	Healthy dolphins		Dolphins with orogenital papillomas		
Variable	Mean	SD	Mean	SD	*p* value
Glucose (mg/dl)	95.01	14.21	93.23	11.31	0.78
Sodium (mEq/l)	155.40	2.36	154.73	1.78	0.21
Potassium (mEq/l)	3.87	0.37	3.84	0.37	0.96
Chloride (mEq/l)	114.28	2.97	113.23	2.91	0.20
Bicarbonate (mEq/l)	21.06	3.59	22.86	3.51	**0.02**[1]
Anion gap	24.05	5.09	22.59	5.72	0.17
BUN (mg/dl)	62.21	8.87	63.59	8.08	0.71
Creatinine (mg/dl)	1.10	0.26	1.08	0.21	0.73
Fibrinogen (mg/dl)	112.65	63.36	92.86	42.68	0.22
Total bilirubin (mg/dl)	0.09	0.04	0.08	0.05	0.26
Direct bilirubin (mg/dl)	0.02	0.04	0.01	0.03	0.21
Indirect bilirubin (mg/dl)	0.07	0.05	0.07	0.06	0.95
Calcium (mg/dl)	9.32	0.40	9.25	0.46	0.27
Phosphorus (mg/dl)	4.94	0.70	4.91	0.57	0.98
Magnesium (mg/dl)	1.45	0.14	1.41	0.15	0.13
Uric acid (mg/dl)	0.68	0.49	0.75	0.46	0.50
Alkaline phosphatase (U/l)	299.83	191.63	275.68	106.50	0.78
Alanine aminotransferase (ALT) (U/l)	40.53	17.60	37.64	9.54	0.65
Aspartate aminotransferase (AST) (U/l)	234.74	62.91	212.91	35.05	0.09
Lactate dehydrogenase (LDH) (U/l)	461.79	63.26	471.55	59.05	0.39
Creatine phosphokinase (CPK) (U/l)	174.14	54.17	171.00	42.80	0.99
Amylase (U/l)	1.63	0.49	1.57	0.32	0.70
Lipase (U/l)	8.60	4.20	7.50	8.03	0.05
Gamma glutamyltransferase (GGT) (U/l)	26.24	4.17	25.86	3.59	0.60
Cholesterol (mg/dl)	155.84	33.53	138.86	25.15	0.05
Triglyceride (mg/dl)	87.58	30.07	82.41	28.10	0.43
Iron (ug/dl)	110.13	37.68	89.91	25.93	**0.03**
WBCs (10^3 cells/ul)	10.13	1.86	10.24	2.22	0.74
Neutrophils (10^3 cells/ul)	3.85	1.00	4.54	1.65	0.11
Band neutrophils (10^3 cells/ul)	0.00	0.02	0.00	0.00	0.63
Lymphocytes (10^3 cells/ul)	2.32	1.20	2.12	0.70	0.75
Monocytes (10^3 cells/ul)	0.28	0.19	0.26	0.18	0.58
Eosinophils (10^3 cells/ul)	3.63	1.51	3.27	1.26	0.34
Basophils (10^3 cells/ul)	0.07	0.11	0.07	0.10	0.95
Erythrocytes (10^6 cells/ul)	3.58	0.22	3.57	0.21	0.64
Hemoglobin (g/dl)	14.36	0.75	14.16	0.91	0.42
Manual hematocrit (%)	40.74	2.27	40.67	2.50	0.96
Mean corpuscular volume (MCV) (fl)	114.07	6.33	114.33	6.04	0.99
Mean corpuscular hemoglobin (MCH) (pg)	40.32	2.27	39.76	2.23	0.34
Mean corpuscular hemoglobin concentration (MCHC) (g/dl)	35.37	1.15	34.86	1.15	0.08
Platelets (10^3/ul)	183.40	43.58	168.05	30.69	0.10

[1]Bold values are statistically significant ($p < 0.05$).

monocytic phagocytosis, and superoxide respiratory burst values were significantly higher in dolphins with orogenital papillomas compared with healthy dolphins. Conversely, the percentage of lymphocytes expressing MHC class II molecules (MHC II+ [%]) was significantly lower in dolphins with orogenital papillomas. MHC II+ (absolute nos.) were also lower in dolphins with orogenital papillomas, although not significantly ($p = 0.07$). No significant differences were found in the remaining immunological tests.

Antibody titers determined in an ELISA system against selected marine bacteria are summarized in Table 3. Dolphins with orogenital papillomas had significantly higher antibody titers to *Escherichia coli*, *Erysipelothrix rhusiopathiae*, *M. marinum*, *V. cholerae*, *V. carchariae*, *V. vulnificus*, and *V. parahemolyticus* as compared with healthy dolphins. Antibody concentrations in dolphins with orogenital papillomas were approximately two-fold higher than those for healthy dolphins for *Escherichia coli*, *Erysipelothrix rhusiopathiae*, *M. marinum*, *V. cholerae*, and *V. parahemolyticus*.

Serum protein electrophoresis data are shown in Table 4. Total globulins, total alpha globulins and alpha-2 globulins were significantly elevated in dolphins with orogenital papillomas compared with healthy dolphins. Gamma globulins were also elevated in dolphins with orogenital papillomas, although not significantly ($p = 0.31$).

Table 2. Immunological test results in healthy dolphins ($n = 86$) and dolphins with orogenital papillomas ($n = 22$)

	Healthy dolphins		Dolphins with orogenital papillomas		
Immune test	Mean	SD	Mean	SD	*p* value
CD2 T cells (absolute nos.)	861.3	531.4	774.0	432.0	0.66
CD4 helper T cells (absolute nos.)	397.0	220.1	355.5	178.0	0.54
CD 19 B cells – Immature (absolute nos.)	569.1	622.1	447.4	225.0	0.88
CD 21 B cells – Mature (absolute nos.)	867.4	917.2	584.8	352.3	0.40
CD2/CD4 ratio	2.24	0.81	2.43	1.36	0.96
CD2/CD21 ratio	1.68	1.34	1.76	1.42	0.54
MHC II+ (absolute nos.)	1,731.7	1,141.3	1,254.2	683.5	0.07
MHC II+ (%)	70.6	17.9	56.1	12.9	**0.001**[1]
T cell proliferation (Con A 2.5)	599.9	419.8	581.8	344.3	0.94
B cell proliferation (LPS 120)	99.3	155.3	173.5	164.2	**0.006**
IgG1 (mg/ml)	11.69	2.62	12.23	2.83	0.33
Granulocytic phagocytosis (%)	20.5	11.7	30.3	12.4	**0.001**
Monocytic phagocytosis (%)	19.2	13.1	28.8	13.6	**0.004**
Natural killer cell activity (100:1)	14.21	12.96	10.17	8.83	0.36
Superoxide production	3.16	1.10	4.24	0.84	**0.0005**
Lysozyme concentration (ug/ul)	6.45	2.55	6.14	1.85	0.97

[1]Bold values are statistically significant ($p < 0.05$).

Table 3. Antibody titers (U/ul) against common marine bacteria in healthy dolphins ($n = 86$) and dolphins with orogenital papillomas ($n = 22$)

	Healthy dolphins		Dolphins with orogenital papillomas		
Antigen	Mean	SD	Mean	SD	*p* value
Escherichia coli	129.9	130.2	250.7	25.9	**0.004**[1]
Erysipelothrix rhusiopathiae	117.8	117.8	218.1	35.2	**0.03**
Mycobacterium marinarum	147.5	156.0	312.6	82.6	**0.0002**
Vibrio carchariae	240.9	85.5	304.0	60.8	**0.006**
Vibrio cholerae	120.8	121.9	231.3	58.1	**0.006**
Vibrio parahaemolyticus	142.7	140.8	281.4	37.1	**0.002**
Vibrio vulnificus	228.5	69.4	269.6	35.6	**0.04**

[1]Bold values are statistically significant ($p < 0.05$).

No significant differences were found in total protein, albumin, alpha-1 globulin, and beta globulin concentrations and albumin/globulin ratio.

Finally, analyses of the recaptured dolphins (*n* = 6) that developed an orogenital papilloma between their first and second captures are found in Table 5. Serum bicarbonate, total alpha globulins, granulocytic phagocytosis, and monocytic phagocytosis were significantly elevated after tumor development. Additionally, antibody titers to the common marine microorganisms *Escherichia coli*, *Erysipelothrix rhusiopathiae*, *M. marinum*, *V. cholerae*, and *V. parahemolyticus* were significantly elevated after tumor development. Serum iron was lower and total globulins, alpha-2 globulins, and B-cell proliferation were higher after tumor development, although not significantly. The percentage of lymphocytes expressing MHC class II molecules (MHC II+[%]) after tumor development was approximately the same as prior to tumor development.

Discussion

Reports of neoplasia are relatively uncommon in bottlenose dolphins. The authors recently reported the first cases of orogenital sessile papillomas in free-ranging bottlenose dolphins which are associated with a gammaherpesvirus and papillomavirus (Bossart et al., 2005; Rehtanz et al., 2006). The tumors appear to be sexually transmitted and are now occurring in epidemic proportions in the

Table 4. Serum protein electrophoresis values in healthy dolphins (*n* =86) and dolphins with orogenital papillomas (*n* = 22)

	Healthy dolphins		Dolphins with orogenital papillomas		
Analyte	Mean	SD	Mean	SD	*p* value
Total protein (g/dl)	7.15	0.45	7.31	0.47	0.35
Albumin (g/dl)	4.52	0.25	4.42	0.25	0.15
Total globulin (g/dl)	2.62	0.54	2.85	0.56	**0.03**[1]
A/G ratio	1.84	0.61	1.62	0.37	0.13
Total alpha globulin (g/dl)	1.25	0.23	1.36	0.15	**0.02**
Alpha-1 globulin (g/dl)	0.40	0.21	0.36	0.15	0.70
Alpha-2 globulin (g/dl)	0.85	0.26	1.01	0.17	**0.02**
Total beta globulin (g/dl)	0.46	0.10	0.47	0.06	0.26
Gamma globulin (g/dl)	1.69	0.54	1.85	0.49	0.31

[1]Bold values are statistically significant ($p < 0.05$).

Table 5. Test results in dolphins that developed orogenital papillomas between first and second capture (*n* = 6)

	Prior to development of orogenital papilloma		After development of orogenital papilloma		
Immune test	Mean	SD	Mean	SD	*p* value
B cell proliferation (LPS 120)	41.1	50.4	224.3	201.8	0.06
MHC II+ (%)	61.1	23.0	66.0	14.0	0.53
Granulocytic phagocytosis (%)	17.8	4.6	33.1	13.4	**< 0.01**[1]
Monocytic phagocytosis (%)	18.1	4.6	33.4	12.0	**0.02**
Escherichia coli[2]	4.7	1.3	234.5	32.3	**< 0.01**
Erysipelothrix rhusiopathiae[2]	3.1	1.1	212.2	26.8	**< 0.01**
Mycobacterium marinarum[2]	8.1	3.0	314.0	92.3	**< 0.01**
Vibrio cholerae[2]	6.0	1.9	220.2	31.9	**< 0.01**
Vibrio parahaemolyticus[2]	5.3	1.2	256.6	32.8	**< 0.01**
Bicarbonate (mEq/l)	18.2	3.5	24.7	1.4	**< 0.01**
Iron (ug/dl)	123.2	27.4	101.2	21.8	0.20
Total globulin (g/dl)	2.7	0.7	3.3	0.4	0.38
Total alpha globulin (g/dl)	1.2	0.3	1.4	0.2	**0.04**
Alpha-2 globulin (g/dl)	0.9	0.1	1.1	0.2	0.06

[1]Bold values are statistically significant ($p < 0.05$).
[2]Antibody titers (U/ul)

CHS and IRL populations (Bossart et al., 2005, 2006; Reif et al., 2007).

Dolphins diagnosed with orogenital papillomas in the CHS and IRL were found to have multiple abnormalities in serum chemical, protein electrophoresis, and immunologic parameters when compared to healthy dolphins. Multiple parameters indicated that dolphins with tumors had an acute-phase inflammatory reaction. Serum iron was significantly lower, and total globulin, total alpha globulin, and alpha-2 globulin were significantly higher in dolphins with orogenital tumors compared with healthy dolphins. Additionally, serum iron was lower, and total globulin, total alpha globulin, and alpha-2 globulin were higher in dolphins with orogenital tumors compared to healthy dolphins from the same populations determined in previous studies (Goldstein et al., 2006). Hypoferremia is a common response to systemic infections or generalized inflammatory disorders (Nemeth et al., 2004). In the acute-phase inflammatory response, serum iron is sequestered by iron-binding proteins, making it unavailable to invading pathogens and thus decreasing the chance for infection (Bossart et al., 2001). Likewise, the hyperglobulinemia with a concurrent alpha-2 hyperalphaglobulinemia in dolphins with tumors also support an acute-phase inflammatory response. Alpha globulins are acute-phase proteins (APPs) that include haptoglobulin, a2-macroglobulin, lipoproteins, and ceruloplasmin and can aid in the detection and quantification of inflammation in humans and domestic animals (Aldridge et al., 2001; Bossart et al., 2001). Further, elevations of APPs have been associated with viral infections in other species; therefore, the association of an acute-phase inflammatory reaction with animals showing viral related orogenital sessile papillomas is not unexpected (Noursadeghi et al., 2005; Parra et al., 2006; Sánchez-Cordón et al., 2007).

Serum bicarbonate was significantly higher in dolphins with orogenital papillomas compared with healthy dolphins. However, the mean bicarbonate values for dolphins with tumors and healthy dolphins in this study were both within the range for healthy dolphins from the same populations determined in previous studies (Fair et al., 2006; Goldstein et al., 2006). Thus, this finding is probably not clinically significant.

A major challenge for the immune system is to recognize and eliminate cells undergoing neoplastic transformation. Immune defense against tumors is complex. It can be mediated early by the innate immune system (i.e., phagocytes, NK cells, cytokines, and complement proteins) and later by the adaptive immune system (i.e., B cells and T cells). The immunological results reported here provide an intriguing glimpse into the innate and adaptive immune responses in dolphins with orogenital papillomas. The increase in granulocytic and monocytic phagocytosis and superoxide respiratory burst values in dolphins with tumors suggests an up-regulated innate immune response, which would be an expected early tumor immune response, especially with viral-associated tumorigenesis (Woodworth, 2002). These data also support the acute-phase inflammatory response postulated above since phagocytes are important effector cells in acute inflammation and produce inflammatory mediators that recruit more inflammatory cells to the site of the infection (Aldridge et al., 2001).

Similarly, the humoral immune component of adaptive immunity also appears to be at least partially up-regulated in dolphins with tumors evidenced by the higher B-cell lymphocyte proliferation values and markedly elevated antibody titers to common marine pathogens. Increased B-cell lymphocyte proliferation has been noted in other gammaherpesvirus infections (Nikiforow et al., 2001; Moser et al., 2005), and increased antibody concentrations have been reported in human papillomavirus (HPV) infections (Bard et al., 2004). These data suggest enhanced innate and humoral immune surveillance in dolphins with orogenital papillomas, which in other species may be associated with viral-induced tumors that express foreign peptides.

The high proportion of dolphins with orogenital papillomas is not surprising. Serologic studies show a high prevalence of antibody to TtPV-2 (Rehtanz et al., unpub. data), a novel papillomavirus isolated from an affected CHS dolphin (Rehtanz et al., 2006). Dolphins are sexually promiscuous with heterosexual and homosexual behavior (Hersh et al., 1990; Wells et al., 2000; Gubbins, 2002). Thus, promiscuous sexual behavior and other close contact would not only serve to transmit the tumors but would expose these dolphins to other pathogens, resulting in the observed immunological changes. This speculation is supported by the adult predilection for tumor development reported previously (Bossart et al., 2005) and the elevated antibody titers to common marine pathogens such as *Erysipelothrix rhusiopathiae*, *M. marinum*, and *V. cholerae*.

Cell-mediated immunity (CMI) is important in viral clearance both in assisting antibody production and in killing infected cells. The only measure of functional CMI in this study (T-cell mitogen-induced LP) showed no significant differences between dolphins with and without tumors. This contradicts data reported in other species with similar tumors. Decreased T-cell mitogen-induced LP studies with associated cell-mediated

immunologic dysfunction have been described in oral hairy leukoplakia (OHL) in humans and cutaneous papillomaviral papillomatosis in killer whales (*Orcinus orca*) and endangered Florida manatees (*Trichechus manatus latirostris*), all disorders that have similar etiopathological features as the dolphin tumors (Bossart et al., 1996, 2002, 2005; Cotran et al., 1999). Studies with humans given an HPV challenge show no change in mitogen-induced T-cell proliferation (Emeny et al., 2002), however, and those with herpes simplex-1 infections also exhibit no change in mitogen-induced T-cell proliferation (Rasmussen & Merigan, 1978). Thus, as in humans with similar viral infections, it appears that the viral classes associated with the orogenital tumors in dolphins do not alter mitogen-induced T-cell proliferation.

CD4+ cells along with CD8+ T cells play important roles in response to viral infections (Koelle et al., 1998; Nikiforow et al., 2001; Emeny et al., 2002; Dobbs et al., 2005). CD4+ cell numbers were not altered in this study, but MHC II expression on lymphocytes in dolphins with tumors, as observed with both percent and absolute numbers of cells, suggests that antigen presentation to CD4+ T lymphocytes could be reduced. MHC II consists of proteins that bind to and present pathogen fragments on the surface of antigen presenting cells for recognition by other immune cells (i.e., CD4+ T cells) (Romano et al., 1992). Although absolute numbers of lymphocytes expressing MHC II were not statistically significant ($p = 0.07$), this reduction may be biologically significant. The lower MHC II expression in dolphins with tumors may represent a partial compromise of the dynamic relationship that MHC II plays in cell-mediated adaptive immunity. In fact, numerous studies have reported reduction or impairment of MHC II expression along with impairment of MHC II processing and presentation pathways with various viral classes, including papillomaviruses and herpesviruses (Tomazin et al., 1999; Scott et al., 2001; Johnson & Hegde, 2002; Neumann et al., 2003). It is worth noting that the MHC and its role in cell-mediated adaptive immunity may be unique in dolphins given its constitutive expression on dolphin T and B cells and warrants further investigation, particularly as it applies to pathogens such as viruses (Romano et al., 1992).

Evaluation of recaptured dolphins prior to and after tumorigenesis provided an opportunity to evaluate hematological, biochemical, and immunological responses prospectively. Despite the small sample size, this prospective approach helps to confirm the suite of changes the authors note in the main analysis and supports speculation that the acute-phase inflammatory response and up-regulated innate and humoral immunity are responses to the tumors and/or the viruses associated with the tumors.

Caution must be exercised in interpreting these data as the immune systems of free-ranging bottlenose dolphins are not well-defined, and the many varied morphologic and functional components of the immune system are only partially defined by the tested variables. Further, this cross-sectional study did not permit adequate ascertainment of the temporal relationship between the parameters measured and papillomas since the main analysis was based on samples taken at a single point in time and the prospective analysis was based on six cases. However, the fact that the orogenital tumors persist and are rapidly increasing in incidence suggests that the tumors can evade immune surveillance despite the presence of antigens that can be recognized by the host immune system (Bossart et al., 2006). It is possible that dolphins with orogenital papillomas may have enhanced innate and humoral adaptive immunity due to increased exposure to other directly transmitted pathogens. It appears unlikely that infection with common pathogens would have occurred selectively among dolphins with papillomas, however. Current studies include re-evaluation of affected dolphins to describe the natural history of the disease and determine whether papillomas may progress to malignant neoplasms as described in captive dolphins (Bossart et al., 2005). Further research to define the complex pathogenesis of dolphin orogenital tumors and to search for other factors that may be responsible for the recent apparent increase in incidence of these tumors is needed as well as continued monitoring of dolphin immune function and health.

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