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SOME SEROLOGIC ASPECTS OF THE IMMUNE RESPONSE IN THE ATLANTIC BOTTLE-NOSED PORPOISE

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Abstract: An investigation of the immunologic responsiveness of *Tursiops truncatus* to *Erysipelothrix insidiosa* and *Clostridium perfringens* Type D antigens was reported. Serum protein changes were recorded by electrophoresis, and the induction time, degree and duration of the immune response were determined by immunofluorescence. Passive protection studies were conducted in mice using porpoise serum dilutions and an LD_{50} of *E. insidiosa* and a TD_{50} of the epsilon toxin of *Cl. perfringens* Type D. Statistically significant quantitative changes in beta-2 globulins were observed in *T. truncatus* following inoculation with either *E. insidiosa* bacterin or *Cl. perfringens* Type D bacterin-toxoid. Newly captured *T. truncatus* possessed circulating antibodies to *E. insidiosa* and *Cl. perfringens* Type D; these antibodies were demonstrated using indirect immunofluorescence. Serum from *T. truncatus* having a 1:1024 titer to *E. insidiosa* prevented the development of clinical signs of disease and bacteremia produced by an LD_{50} of *E. insidiosa* in mice.

INTRODUCTION

The role of serology in diagnosis of disease is an important aspect of evaluating disease problems and formulating proper disease prevention procedures. An understanding of the immune response in *Tursiops truncatus* is essential to preventing certain infections. There are no known reports of electrophoretic studies on serum protein changes related to the development of an immune response in porpoises. There are numerous reports of *Erysipelothrix insidiosa* infections in porpoises.[2,6,9,10] Recently an evaluation of various *E. insidiosa* immunogens was reported; however, there are no known reports of protective capabilities of antibodies produced in porpoises.[3] In addition, there are no reports of *Clostridium perfringens* Type D intoxication in porpoises. This organism has been isolated from *T. truncatus* at necropsy (R. J. Hidalgo and R. B. Simpson. 1969. Personal communication) and from two other marine mammals, *Globocephalus melaena* (G. W. Klontz. Unpublished data) and *Orcinus orca*.[5] Enterotoxemia and clostridial hepatoxic encephalopathy syndromes have been associated with the organism in these species respectively. The similarity of these syndromes to those attributed to epsilon toxin provided the basis for considering *Cl. perfringens* Type D.

The primary objective of this study, therefore, was to describe certain serologic aspects of the primary immune response of the Atlantic bottle-nosed porpoise, *T. truncatus*, to *E. insidiosa* and *Cl. perfringens* Type D antigens.

METHODS AND MATERIALS

The primary immune response study was conducted in five adult newly captured *T. truncatus*. Three animals were

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inoculated subcutaneously with 2 ml doses of *E. insidiosa* bacterin[1] and four were inoculated subcutaneously with 5 ml doses of *Cl. perfringens* Type D bacterin-toxoid[2] (Table 1). Blood samples were taken preinoculation and postinoculation on days 7, 14, 21, 35, and 49. Serum protein profiles pre- and postinoculation were recorded by zone electrophoresis using cellulose polyacetate strips.[3] Serum protein components were measured electronically[4] and total serum protein was measured by refractometry.[5] Induction time and degree and duration of the immune response were determined by indirect immunofluorescence (IF).[1]

Passive protection studies were performed in adult albino mice, using serum dilutions from all porpoises in the study, mixed with an ID_{50} dose of *E. insidiosa* or a TD_{50} dose of the epsilon toxin of *Cl. perfringens* Type D. The ID_{50} was determined to be 0.1 ml of a 1:2032 suspension of *E. insidiosa* while the TD_{50} was determined to be 0.1 ml of a 1:68 dilution of cell-free culture fluid from a growth of *Cl. perfringens* Type D.

Electrophoretic and immunofluorescence data were evaluated statistically using analysis of variance with the random block design.[8] A null hypothesis was used to test independence of antibody production to either an increase or decrease in total serum proteins, gamma globulin concentrations, beta-2 globulin concentration, and IF titers.

RESULTS

Serum protein measurements from the primary immune response study were tabulated (Table 2). Preinoculation measurements are listed as Day 0. Homogeneity of preinoculation serums is suggested by variance of total protein (0.000425), gamma globulin (0.0525), and beta-2 globulins (0.0099).

Among porpoises receiving *E. insidiosa* antigen, antibody production was independent of changes ($P>5\%$) in total protein of gamma globulin concentrations. Significant changes ($5\%>P>2.5\%$) in beta-2 globulin concentrations were observed among these animals however.

TABLE 1. Primary immune response antigenization schedule.

Porpoise	*Cl. perfringens* D. bacterin-toxoid	*E. insidiosa* bacterin	Dose	Route
1		**	2 ml	S.C.
2	***		5 ml	S.C.
3	***		5 ml	S.C.
4	***		5 ml	S.C.
		**	2 ml	S.C.
5	***		5 ml	S.C.
		**	2 ml	S.C.

S.C. = Subcutaneous

[1] Norden Laboratories, Lincoln, Nebraska.
[2] Norden Laboratories, Lincoln, Nebraska.
[3] SepraTek System, Gelman Instrument Co., Ann Arbor, Michigan.
[4] Densicord 542, Photovolt Corp., New York, New York.
[5] TS Meter, American Optical Co., Dallas, Texas.

TABLE 2. Weekly fluctuations postinoculation in serum proteins of **T. truncatus** (in grams/100 ml and A/G ratio).

Animal	Total Protein	Albumin	Beta-2	Gamma	A/G Ratio
1*					
Day 0	7.5	4.35	.30	1.20	1.38
Day 7	7.0	4.07	.36	1.26	1.00
Day 14	7.2	4.59	.24	1.12	1.76
Day 21	8.4	5.00	.50	1.50	1.47
Day 35	8.2	3.48	.50	2.24	.74
Day 49	8.0	4.90	.40	1.22	1.57
2**					
Day 0	7.5	3.82	.55	1.23	1.04
Day 7	11.7	6.01	.83	2.18	1.06
Day 14	9.2	5.07	.94	1.12	1.23
Day 21	13.8	7.51	1.23	2.26	1.19
Day 35	6.3	3.15	.66	1.32	1.00
Day 49	12.7	7.56	.69	2.24	1.47
3**					
Day 0	7.7	4.24	.43	1.56	1.23
Day 7	9.3	5.00	.47	1.67	1.17
Day 14	8.0	4.39	.53	1.49	1.22
Day 21	9.0	5.24	.59	1.68	1.39
Day 35	8.2	4.70	.60	1.50	1.34
Day 49	7.3	4.02	.55	1.46	1.22
4***					
Day 0	8.0	4.27	.52	1.66	1.14
Day 7	9.2	5.05	.49	1.62	1.22
Day 14	6.3	3.67	.47	1.03	1.39
Day 21	9.1	4.77	.76	1.52	1.10
Day 35	8.1	4.32	.43	1.62	1.14
Day 49	9.4	5.16	.57	1.95	1.22
5***					
Day 0	7.7	4.19	.49	1.66	1.19
Day 7	7.7	4.03	.48	1.66	1.10
Day 14	7.7	4.08	.47	1.63	1.12
Day 21	8.8	4.53	.55	2.06	1.06
Day 35	7.7	4.32	.41	1.76	1.28
Day 49	6.5	3.34	.35	1.23	1.06

* *E. insidiosa* bacterin only
** *Cl. perfringens* Type D bacterin-toxoid only
*** both antigens received

Among porpoises receiving *Cl. perfringens* Type D antigen, antibody production was independent of changes ($P>5\%$) in total protein or gamma globulin concentrations. Significant changes ($P<0.5\%$) in beta-2 globulin concentrations were observed in these animals however.

The only serum fraction demonstrating a significant change over a period of time was the beta-2 globulin in porpoises receiving *Cl. perfringens* Type D ($5\%>P>2.5\%$). Changes in beta-2 globulin concentrations over a period of time in porpoises receiving *E. insidiosa* were not significant ($P>5\%$).

Results of IF titrations from the primary induction study were tabulated (Table 3). Preinoculation antibody titers were demonstrable in each antigenized group of *T. truncatus*. Titers against *E. insidiosa* ranged from 1:8 to 1:128, and those against *Cl. perfringens* Type D ranged from 1:2 to 1:128. Antibody

TABLE 3. Results of immunofluorescence titrations of anti-organism serums from the primary immune response induction study.

Porpoise	Day	High IF Dilution *Cl. perfringens* D	High IF Dilution *E. insidiosa*
1	0		1:32
1	7		1:32
1	14		1:64
1	21		.....
1	35		1:256
1	49		1:128
2	0	1:64	
2	7	1:128	
2	14	1:64	
2	21	1:32	
2	35	1:32	
2	49	1:32	
3	0	1:128	
3	7	1:512	
3	14	1:256	
3	21	1:512	
3	35	1:512	
3	49	1:512	
4	0	1:2*	1:8
4	7	1:32	1:256
4	14	1:128	1:512
4	21	1:256	1:256
4	35	.....	.....
4	49	1:64	1:512
5	0	1:32	1:128
5	7	1:128	1:256
5	14	1:256	1:128
5	21	1:64	1:128
5	35	1:128	1:512**
5	49	1:256	1:128

* This dehydrated serum sample was reconstituted with 0.2 ml. pH 7.2 phosphate buffered saline prior to standard dilution.

** 1:256 was negative; 1:128 was positive.

production was shown to be related to IF titer changes among animals receiving either antigen ($P<0.5\%$), but over a period of time such changes were not significant ($P>5\%$).

All porpoises had demonstrable IF titers through day 49. The highest anti-*E. insidiosa* and anti-*Cl. perfringens* Type D IF titers on day 49 were 1:512, and the low titers were 1:128 and 1:32 respectively.

Passive protection studies indicated that porpoise serum having a 1:1024 IF titer to *E. insidiosa* prevented the development of clinical signs of disease and bacteremia produced by an ID_{50} of *E. insidiosa* in mice (Table 4). One mouse protective unit was determined to be 0.1 ml of a 1:4 phosphate buffered saline dilution of serum from a porpoise having a 1:1024 anti-*E. insidiosa* IF titer. A second study utilizing porpoise serum with a 1:256 IF titer failed to protect mice.

A similar passive protection study using porpoise anti-*Cl. perfringens* Type D serum yielded inconclusive results (Table 5). The highest dilution of porpoise serum protecting two of three mice for a 24 hour observation period was 1:16, however two mice died at 1:2 and all three mice in the TD_{50} control died within the same time period.

TABLE 4. Results of the passive protection study in mice to **E. insidiosa.**

Porpoise Serum Dilutions	No Clinical Signs	Conjunctivitis	Rough Hair Coat	48 Hour Bacteremia
1:1	3/3	0/3	0/3	0/3
1:2	3/3	0/3	0/3	0/3
1:4	2/3	0/3	1/3	0/3
1:8	1/3	0/3	2/3	0/3
1:16	0/3	2/3	3/3	1/3
1:32	0/3	3/3	3/3	2/3
1:64	0/3	3/3	3/3	2/3
ID_{50} (Control)	0/3	3/3	3/3	2/3

TABLE 5. Results of inoculating mice with **Cl. perfringens** Type D toxin-antitoxin.

Porpoise Serum Dilutions	#Mice dead 7 hours	24 hour mortality ratio
Undiluted	1	1/3
1:2	2	2/3
1:4	1	1/3
1:8	1	1/3
1:16	1	1/3
1:32	2	2/3
1:64	2	2/3
PBS control	0	0/3
Toxin (TD_{50})	3	3/3

DISCUSSION

Changes in both total protein and gamma globulin concentrations were observed during the sampling period, yet they were not statistically significant. Possibly this can be explained by such factors as physical stresses involved in capture and adaptation, dehydration, inappetence, and environmental fluctuations. Any one or a combination of these factors could exert an interacting influence and account for the lack of significance.

The finding of significant quantitative changes in beta-2 globulin concentrations among animals despite interacting influences further supports the conclusion that antibody production is not independent of a change in this fraction. Also, quantitative changes in this fraction are not reported as being typically associated with the factors mentioned, and are more consistently associated with immunization procedures. Lack of statistical significance of changes in beta-2 globulins over a period of time in porpoises receiving *E. insidiosa* antigen can best be explained by inadequate sample size. Despite the lack of significance, the response over a period of time was similar to that observed in animals receiving *Cl. perfringens* Type D antigen, whose changes were significant.

The presence of preinoculation IF titers to both antigens plus the short latent period and the rapid rise in titers in each porpoise in the primary induction study is characteristic of a secondary immune response in mammals. The wide range of preinoculation titers (1:2-1:128) within each group of antigenized porpoises explains the different responses to an antigen within a test group.

Attempts to statistically assess positive or negative correlation between beta-2 globulins and IF titers was not possible due to preinoculation IF titers and insufficient data. Time since last exposure and the type and dose of antigen could not be determined with regard to preinoculation titers.

Natural exposure to *E. insidiosa* and *Cl. perfringens* Type D in free-living *T. truncatus* is supported by the presence of preinoculation IF titers to these two antigens. The ubiquitous nature of *Cl. perfringens* is well documented, and numerous cases of erysipeloid in fishery workers and the occurrence of *E. insidiosa* in fish have been documented.[6,7,11] Experimental transmission of *E. insidiosa* by houseflies and blood sucking arthropods suggests another possible route of infection for porpoises.[2] Open wounds, often the result of over-zealous play among porpoises, together with the insects which may be in the food preparation areas should be considered in the epizootiologic analysis of erysipelas in porpoises.[2]

Although the presence of natural antibodies reacting with these two antigens was not conclusively ruled out, the increased beta-2 globulin fraction does not support this consideration. Nearly all natural antibodies against bacterial and other cellular antigens in mammals belong to the immunoglobulin M class of gamma globulins.[4]

Protective capabilities in the toxin neutralization study using porpoise anti-*Cl. perfringens* Type D serum were neither substantiated nor negated. Inaccurate measurement of volumes of toxin or antiserum dilutions, or variability of affinity of the antitoxin to the toxin preparation used could account for the inconsistencies.

The ability of porpoise anti-*E. insidiosa* serum to prevent the development of clinical signs of disease and becteremia produced by an ID_{50} of *E. insidiosa* in mice was demonstrated. This certainly suggests that circulating antibodies to *E. insidiosa* in porpoise serum will protect porpoises as well.

CONCLUSIONS

The investigation permitted the following conclusions:

1. Significant quantitative changes in beta-2 globulins were observed in *T. truncatus* following inoculation with either *E. insidiosa* bacterin or *Cl perfringens* Type D bacterin-toxoid.

2. Free-living *T. truncatus* may and do possess circulating antibodies to *E. insidiosa* and *Cl. perfringens* Type D.
3. The indirect immunofluorescent staining technique was successful in demonstrating circulating antibodies in *T. truncatus* serum to *E. insidiosa* and *Cl. perfringens* Type D.
4. *Tursiops truncatus* serum having a 1:1024 immunofluorescent titer to *E. insidiosa* prevented the development of clinical signs and bacteremia produced by an ID_{50} dose of *E. insidiosa* in mice.

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