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THE IMMUNOLOGY OF GREEN TURTLE FIBROPAPILLOMATOSIS

by

Rene' A. Varela

A Thesis Submitted to the Faculty of

The College of Science

in Partial Fullfillment of the Requirements for the Degree of

Master of Science

Florida Atlantic University

Boca Raton, Florida

December 1997

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Rene' A. Varela

This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Peter Lutz, Department of Biology, and has been approved by the members of his supervisory committee. It was submitted to the faculty of The College of Science and was accepted in partial fullfillment of the requirements for the degree of Master of Science in Biology.

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ABSTRACT

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Title: The Immunology of Green Turtle Fibropapillomatosis

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Green Turtle Fibropapillomatosis has been a disease of intense investigation, with little ascertained as to its cause. This investigation thus delved into the possible link between this disease and immune compromise. This was achieved by creating three groups of animals, one characterized as healthy another as diseased, and a third as recovered. Blood taken from each animal was then applied to the four aspects of our investigation: general chemistry, hematology, specialized chemistry, and in vitro cell proliferation to establish the best diagnostic cues. General chemistry, hematology, and specialized chemistry proved to be inconsequencial in elucidating the effects of possible immune compromise. In vitro cell proliferation, though, proved conclusively that t and b like cells were severely depressed in diseased and "recovered" animals, giving conclusive evidence of immune dysfunction in association with fibropapillomatosis.

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INTRODUCTION

Since the first published case of green turtle fibropapillomatosis (GTFP) in the late 1930's(Smith and Coates, 1937), its incidence has been increasing dramatically to such levels that in some areas of the world, greater than 50 percent of green sea turtle populations have been effected (Herbst, 1994). These rising numbers poses a significant problem in the all-ready hampered management efforts of this endangered species. This is a disease that is severely debilitating, characterized by the formation of tumors throughout the animal's body (Herbst, 1994). The primary effect of GTFP is that of a physical nature, in that vision may be occluded, movement impeded, digestion obstructed, and breathing capacity reduced from the physical presence of these masses; ultimately reducing this animal's ability to forage for food and avoid predators. Affected animals are reduced to nonviable individuals. The all ready reduced numbers of these animals coupled with the mortality of this disease has thus made evident that the source of this disease must be found if the green sea turtle (*Chelonia mydas*) is to stand a chance of survival as a species.

Tumor masses vary in number and size amongst individuals, with a maximum size of approximately 10 cm, and have been found in almost all tissues. They are most often seen on front flippers and

neck, periocular tissues, plastron, carapace; occasionally on the cornea, in the lungs, liver, kidneys, and gastrointestinal tract (Campbell, 1996). A pathology report* described the tumors as: "... raised sessile masses. The tumors consist of abundant fibrous connective tissue thrown up into multiple fibrovascular papillary projections, which are lined by hyperplastic hyperkerototic squamous epithelium. The stratum basalis has mild multifocal dysplasia. There is mild melanophage incontinance with multifocal stromal granular golden-brown melanin deposition. Occasional papillomas contain mild to moderate numbers of spirochid trematode ova. Currently, these tumors are considered due to a viral infection. However, viral inclusions are not seen. Recurrence is possible."

In spite of a considerable effort, to date there has been little or no success in finding the cause of this disease. Even some more recent work was based on strictly correllary scenarios, such as a connection with the animals' normal parasite fauna (Griener, 1992). And although one recent investigation appeared to indicate the involvement of a non-filterable agent in the form of a virus in the etiology of this disease (Klein et al, 1993), there is no conclusive confirmation of a virus as the causative agent of GTFP. One major handicap in investigating GTFP is that almost nothing is known about the sea turtle immune system. The intent of this study was to provide information on the immune system of healthy sea turtles and investigate the degree of immunological compromise shown by GTFP afflicted turtles. To assist in this investigation, the aspects of

* University of Miami School of Medicine, Department of Comparative Pathology. Pathology report. Submitted November 1996.

immune function will be rooted in the knowledge gained through mammalian studies as well as what has been learned about other reptiles.

Classification of the immune response can be divided into two primary responses, the humoral response and the cell-mediated response (Fig. 1). The humoral response is determined by the types and amount of specific and non-specific antibody that can be produced against a given antigen. It is characteristically the first response, with the antibody remaining in the serum for a period beyond a given immune episode. The cell-mediated response, on the other hand, is determined by the type and amount of specific immune cell populations which can be produced in response to a wide range of pathogens. Both responses are essentially driven by the activity of specific immune cells called leukocytes. These leukocytes are of several different cell types, distinguished by morphology and activity; these are lymphocytes, neutrophils, eosinophils, monocytes, and basophils (Fig. 2).

Lymphocytes are the chief immune cells in both mammals and reptiles, conveying specificity, diversity, memory, and self/nonself recognition which is carried out through the subpopulations of B and T lymphocytes. A comparable heterogeneity also exists in teleost fishes, alligators, and reptiles (Cuchens et al, 1976). The B lymphocytes are typically the antibody producers as well as antigen presenting cells; they essentially tweak the T lymphocytes and mediate the humoral immune response. T lymphocytes are the primary component of the cell-mediated immune response, with two

distinct subpopulations emerging in the form of T cytotoxic (T_C) and T helper (T_h) cells. T helper cells are relegated to cytokine production when stimulated antigenically by antigen presenting cells which present portions of a specific antigen with the MHC molecule (Fig. 1). These cytokines, in turn, activate B cells, T_C cells, macrophage, and various other cells to propagate and migrate to specific sites. Cytokine activated T_C cells transform to cytotoxic T lymphocytes (CTL) which then have the ability to destroy virus infected cells (Fig. 2). In both mammals and reptiles the number of circulating lymphocytes can decrease in response to malnutrition, and lymphocytosis can occur in response to inflammation, wound healing, parasitic infection, and viral disease. While normal lymphocyte counts may vary amongst individual species, overall, reptilian lymphocytes have a very similar function to those of mammals. It is also apparent that the major classes of lymphocytes are B and T cells, that are involved in a variety of lymphocytic functions. The B cells produce several types of immunoglobins and the different subclasses of T cells moderate the immune responses.(Kuby, 1991; Campbell,1996)

Monocytes generally occur in low numbers in the blood of both mammals and reptiles; they grow and integrate into tissues to later become macrophage. These larger, more active cells have an increased phagocytic ability, increased levels of lytic enzymes, and secrete a variety of soluble factors; all aspects which make them prime antigen presenting cells. They are activated by phagocytosis, cytokines produced by activated T_h cells, mediators of inflammatory

response, and bacterial cell wall products. In reptiles, they participate in inflammatory responses and play an active role in granuloma and giant cell formation, particularly in granulomatous responses to bacterial infections and ova of spirorchid trematodes. They also participate in antigen specific immunoglobulin interactions, especially involving IgM and IgY. It is thus noted that peripheral blood monocyte counts increase following antigenic challenge and with infectious diseases.(Kuby, 1991)(Campbell,1996)

Neutrophils are second only to lymphocytes in percentage of total white blood cell count. These are also phagocytic cells, with a higher bacterial killing capacity than macrophage, and are generally the first cells to arrive at the site of inflammation. In fact, increased numbers of circulating neutrophils, leukocytosis, is used to indicate the presence of infection. In reptiles, these cells are known as heterophils with all the same functional characteristics as their mammalian counterparts. As with mammalian neutrophils, significant increases in numbers of heterophils are associated with inflammatory diseases. An increase in heterophils can also be associated with noninflammatory disorders such as stress, neoplasia, and myeloid leukemia. (Kuby,1991; Campbell,1996)

Eosinophils, like neutrophils and heterophils, are motile phagocytic cells. Their major role is in defense against parasitic organisms by release of the contents of their eosinophilic granules, damaging the parasite membrane. Reptiles exhibit an influence in eosinphilic number by parasitic and nonspecific stimuli. In fact, turtle eosinophils have been shown to phagocytize immune

complexes, indicating their participation in immune responses. (Kuby, 1991)(Campbell,1996)

In contrast to the other leukocytes, basophils are not phagocytic immune cells. They play the role of allergic responders, by releasing histamine from their dense granules, which is the primary mediator of the allergy cascade. With reptiles, the number of circulating basophils may increase with certain blood parasites such as heamogregarines, trypanosomes, and pirheamacyton iridovirus infections. (Kuby,1991; Campbell,1996)

It is the interaction of all these cells, mediated by the activity of the T_h cells, which determines the strength and type of response the immune system can mount. In fact, it can be said that T cells play a central role in the immune system such that a T cell deficiency can affect both humoral and cell mediated responses. The absence of T_h cells in this circumstance, and its abrogated role as B cell activator, would then lead to decreased antibody production. In fact spleenectomized, thus T cell limited, lizards show a total loss of serum antibody response (Kanakambika et al, 1972). In instances of congenital T cell dysfunction, the impact on the cell mediated system may be severe causing reduction in delayed type hypersensitivity response and cell mediated cytotoxicity. Whereas defects in the humoral system are associated primarily with infections by encapsulated bacteria, defects in the cell mediated system are associated with increased susceptibility to viral, protozoan, and fungal infections. The severity of the defects may be such that

infections with viruses that are rarely pathogenic for normal individuals may be life-threatening. (Kuby, 1991)

Evidence also exists for non-congenital sources of a similar immune compromise. AIDS research uncovered a viral etiology as causing diminished T_h cell counts and the associated diseases of unusual occurrance. Also discovered was a diminished T lymphocyte proliferation and T helper cell responsiveness (Amadori A, 1988; Clerici M, 1992). Environmental contaminants have been directly connected with T lymphocyte dysfunction through impaired delayed type hypersensitivity and serum antibody responses in captive harbor seals (Ross et al, 1994) and wild beluga whales (DeGuise S, 1995). Papilloma tumors have been documented in a variety of animals including sperm whales (Lambertsen R, 1987). A T cell deficiency associated with persistent papillomatosis in a young bull has also been documented (Duncan et al, 1975). Despite the lack of a singular source, the outcome of T cell compromise clearly appears to be consistant: weakened individuals suffering from diseases which normally would not be pathogenic. This is a model which would seem to fit well with green turtle fibropapillomatosis, as most individuals are weakened by secondary infections and amplified parasite burden (Moretti R, personal communication).

There is every indication that green sea turtles may have an immune system similar to that of other vertebrates and its reptilian relatives, with similar leukocytes and a lymphocyte heterogeneity. These lymphocytes could then be stimulated to proliferate *in vitro* as in standard mammalian treatments (Kurnick, 1995). In fact,

preliminary work on the green sea turtles has indicated this possibility(McKinney C & Bentley T, 1985). This will produce an invaluable tool in comparing the immune status of healthy versus diseased turtles and indicating the source of any immune dysfunction that may be in operation. Supporting evidence will be provided by conducting serum chemistry, hematology, serum protein electrophoresis analyses. As a result, a much clearer profile of fibropapillomatosis will be achieved on a clinical level.

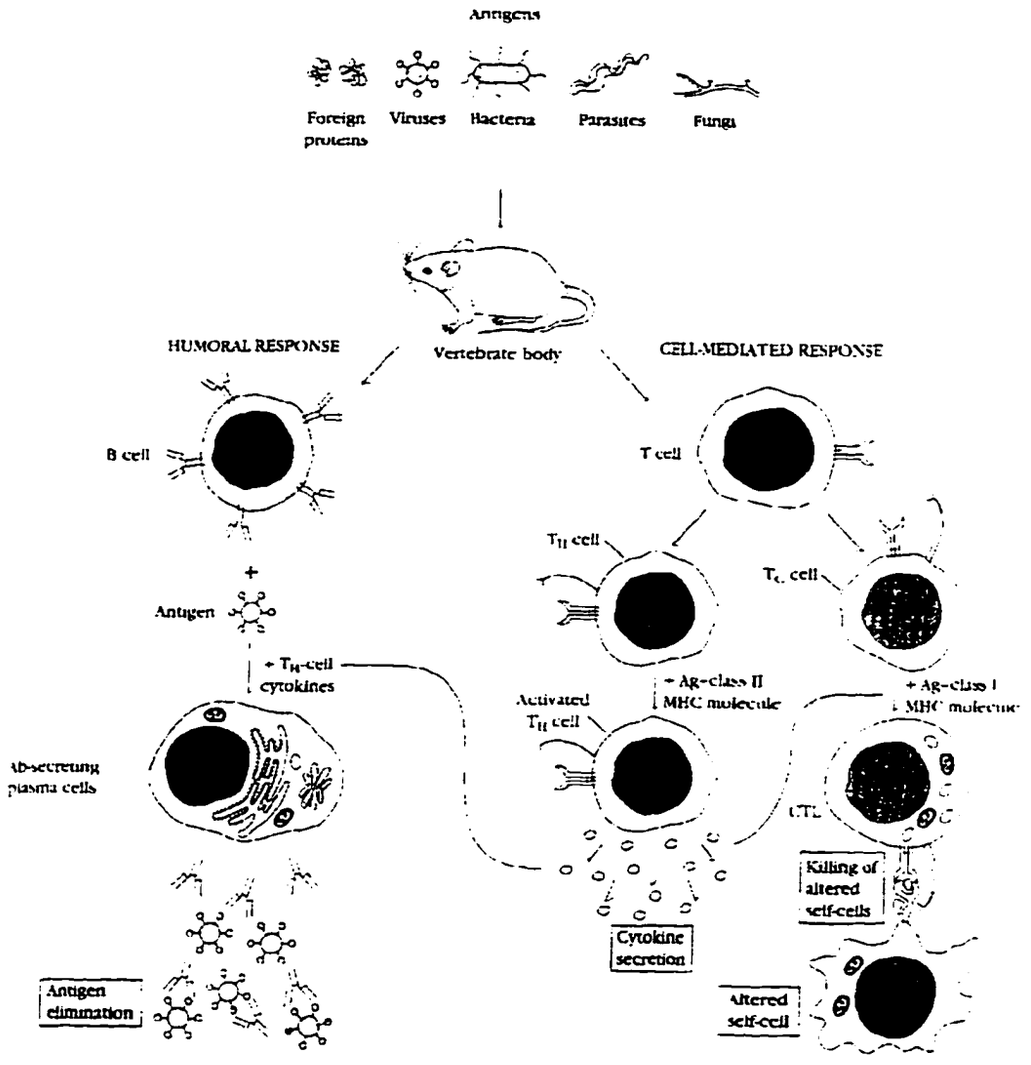

Figure 1: The Humoral and Cell-mediated Responses (Kuby, 1991)

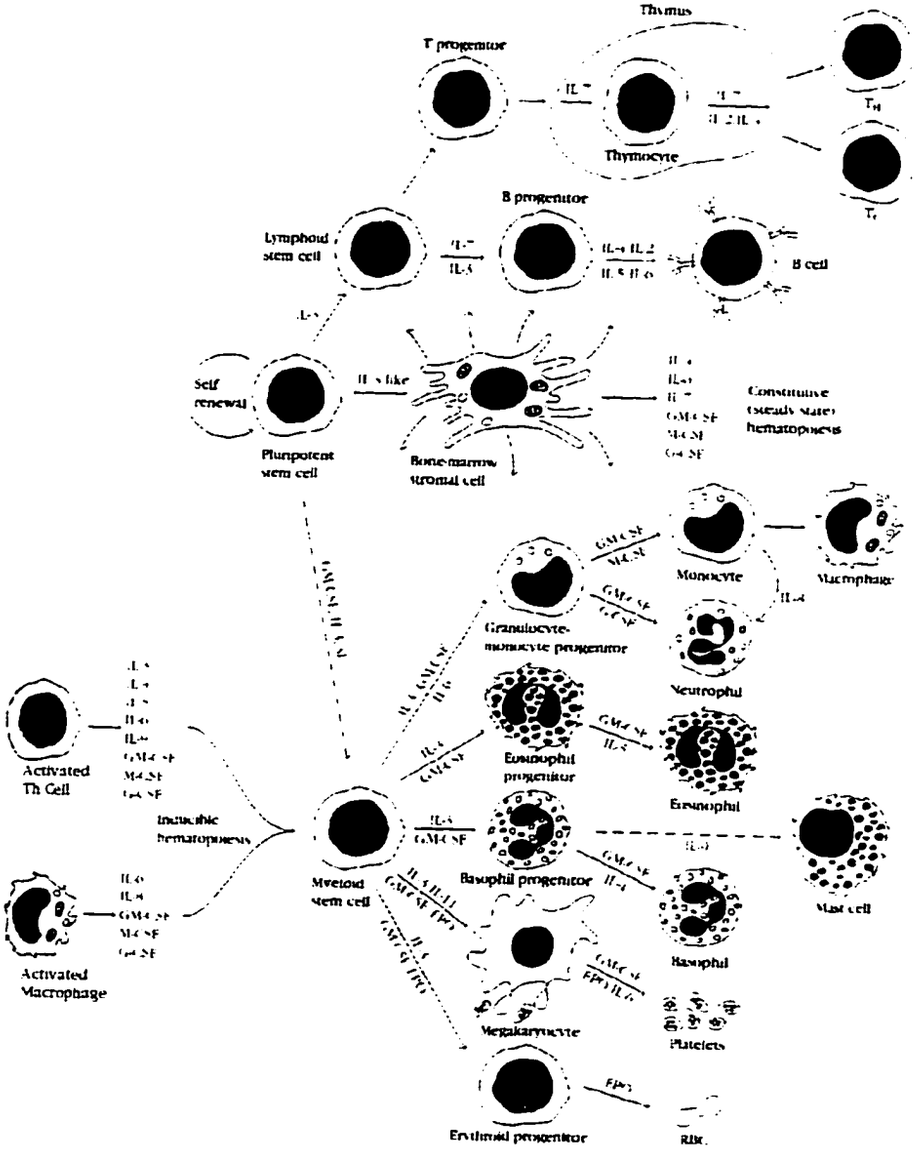

Figure 2: Leukocyte Hematopoesis
(Kuby, 1991)

MATERIALS AND METHODS

Green sea turtles (*Chelonia mydas*) were used for this study and categorized into two major groups: a group (N group) of adult and sub-adult animals in long term captivity at the Miami Seaquarium with no history of exposure to or contraction of GTFP, and a group (P group) consisting of juvenile animals that have been or will be treated for tumors associated with GTFP at the Turtle Hospital in Marathon, Florida. Within the P group, there was a diseased group consisting of animals with the most recent episodes of tumor growth, and a group of healthy animals which had tumors at one point but have shown no signs of regrowth and are considered ready for release. In both the Miami Seaquarium and the Turtle Hospital, the turtles were kept in pools supplied with sand filtered local seawater, and fed a controlled diet of Purina Chow and some squid.

Approximately 3-6 ml. of blood was drawn from the dorso-lateral neck venous sinus of each animal using a 3-6 cc. unheparinized syringe with 21 ga. needle. The blood was immediately transferred to a heparin coated 3cc vacutainer collection tube, with several drops reserved for immediate whole blood slide preparation. The samples was then transported in a cooler containing an ice pack (kept out of direct contact with the samples) back to the

lab in the Department of Comparative Pathology at the University of Miami School of Medicine for processing. Once there, a small portion of each sample was drawn into microcapillary tubes for packed cell volumes. Another 300µl was reserved from each sample in microtainer separator tubes for clinical chemistry analysis, additional hematology values, and serum protein electrophoresis. The remaining volume was used for *in vitro* lymphocyte proliferation assays.

The clinical chemistry analysis was performed as a UM Advanced Avian Panel on both Kodak Ektachem and Dupont Analyst Chem14 systems (with total protein assessed by refractotmeter), calibrated to the Comparative Pathology Lab avian standards. Resultant values were then analyzed and sorted as individuals in each of the three previously described groups. For the individual groups each value's mean and standard error was calculated and one-way anovas performed.

Hematology assays were run as UM avian CBC's. A packed cell volume was derived from centrifuging blood drawn into microcapillary tubes, and reading the packed cell volume as a percentage of total blood volume. White blood cell differentials were then assessed by first staining blood smear slides made at the time of bleeding in Diff Quik differential stain. The cells were counted under 100x magnification and direct numbers of lymphocytes, monocytes, eosinophils, basophils, and heterophils will be represented as a percentage out of a hundred cells counted. Both white blood cells and red blood cells from whole blood were then

counted using the Unopette #5877 and Unopette systems (Becton-Dickinson, Rutherford, NJ) and hemocytometer under 10x's and 40x's magnification respectively. White blood cells were represented as total number counted in an entire chamber. This value was then divided by the sum of the differential percentages of heterophils and eosinophils and multiplied by 3520, to attain an adjusted total white blood cell count per ml of blood. Red blood cells counted were multiplied by 10000 to attain total red blood cells per ml of blood. All values were analyzed for the individuals and segregated into the three test groups. To each group a mean ,standard error, and one-way anovas were calculated for all the categories.

Protein electrophoresis was performed using the Beckman Paragon Electrophoresis System, including the SPE II protein electrophoresis kit, electrophoresis cell, dryer, and densitometer for evaluating the gel. Calculations performed by the densitometer were derived from areas under each scanned peak and represented as percentages of total serum protein which was attained previously from refractometry. Values represented were that of total protein, albumin %, α-1 globulin %, α-2 globulin %, β-globulin %, γ-globulin %, and albumin/globulin ratio. For each test group means, standard errors, and one-way anovas were calculated on each value.

In vitro lymphocyte proliferation began by isolating the lymphocytes from whole blood via Histopaque-1077 (Sigma co.). The cell yield for each individual were then determined by staining 10 μl of the cell solution with 200 μl of Trypan Blue and counting in a hemocytometer under 10x magnification; this value is an exact count

in millions of cells. Each sample was then diluted accordingly with complete media to bring the concentration of lymphocytes to $1x10^6$/ml. The lymphocytes were then exposed to classic immunoblastic stimulants in varying concentrations to attain differential stimulation according to concentration as well as lymphocyte sub-population. For the purposes of this experiment treatments were of varying concentrations of PMA, ionomycin, concanavalin A, phytohemagglutinin, pokeweed mitogen, lipopolysaccharide, and complete media as a control, with each treatment having been performed in triplicate. The cells were first plated on 96 well flat bottom cell culture plates at 100 µl per well in enough wells to perform each treatment in triplicate. Then 100 µl of prepared stimulants or media were then added to the appropriate wells. The plates were then incubated at 37° C for 48 hours. Each well was then pulsed with 25 µl of ^{3}H-thymidine, and set to incubate at 37° C for 24 more hours. The plates were then removed from the incubator and the cells harvested using the Wallac 1205 Betaplate liquid scintillation counter. All treatment cell counts per individual (after averaging triplicates) will be normalized by dividing by cell counts under treatment with complete growth media; this will give a stimulation index for each treatment. These stimulation indices will then be analyzed according to test group, deriving means, standard errors, and one-way anovas for each group.

RESULTS

Clinical Chemistry

Clinical chemistry values were determined for healthy (n=27), diseased (n=22), and recovered (n=8) turtles; these values are shown in Table 1. Noteworthy, was the wide range for normal "healthy" turtles, for example blood urea nitrogen (BUN) had a range of 6-112mg/dl and glucose had a range of 57-501mg/dl. The healthy range completely encompasses the means and ranges of the other two test groups. This same trend is noticed with all the other chemistry values with the exception of those cases such as bilirubin, phosphorus, and uric acid when no difference is noticed between the means themselves.

Hematology

Complete blood counts were determined for healthy (n=27), diseased (n=22), and recovered (n=8) turtles. A trend of increasing white blood cells was noticed for the diseased group, but one-way anova revealed no significant difference between any of the groups in this respect (Figure 3). Figure 4 depicts the relative percentages and types of leukocytes found in the blood of turtles in each group. In this case one-way anovas showed significant differences between healthy/diseased also recovered/diseased lymphocytes and heterophils. Between healthy and recovered animals no significant

difference was noticed for lymphocytes and heterophils. Differences between the test groups in the other leukocytes are not apparent. Lymphocyte/heterophil ratios for healthy, diseased, and recovered turtles were then determined to be 0.73, 0.48, and 0.86 (Figure 5). One-way anova revealed significant differences between healthy /diseased also recovered/diseased, while healthy/recovered showed no difference.

Serum Protein Electrophoresis

Serum protein electrophoresis was performed for healthy (n=27), diseased (n=22), and recovered (n=8) turtles. The most interrelated serum proteins appeared to be albumin and gamma globulin, with one-way anova showing significant differences arising for healthy/diseased also recovered/diseased for both proteins (Figure 6). The other serum proteins showed very minor variation amongst the test groups. Albumin/globulin ratios for, presented in Figure 7, were determined to be 0.88, 0.49, and 0.80 for the the groups respectively. Significant differences were apparent for healthy/diseased as well as recovered/diseased turtles, while no difference was noticed for healthy/recovered.

In Vitro Lymphocyte Proliferation

In determining optimal lymphocyte proliferation for healthy (n=16), diseased (n=22), and recovered (n=8) turtles, various concentrations were used for each type of lymphocyte stimulant. It was seen that PMA, at a concentration of 25nM had to be coupled with ionomycin at a concentration of .3nM for the best stimulation to occur (Figure 8). The best stimulation using ConA was evoked with a

concentration of 10μM/ml (Figure 9). The best concentration for PHA proved to be 20μg/ml (Figure 10). Pokeweed mitogen elicited its best response at 1.25μg/ml (Figure 11). A concentration of 50μg/ml performed the best for lipopolysaccharide (Figure 12). United on one graph are all the best concentrations of each lymphocyte stimulant (Figure 13). Significant differences were noticed between the healthy and the other two groups for all the stimulants. One-way anova proved the differences for healthy/diseased and healthy/recovered in the case of PMA/ionomycin, ConA, PHA, PWM, and LPS to be quite distinct. At the same time, there was no significant difference between the diseased and recovered groups for PMA/ionomycin , ConA, PHA, PWM, and LPS.

	HEALTHY		DISEASED		RECOVERED	
	Mean	Range	Mean	Range	Mean	Range
Glucose*	112.1	57-501	103.8	74-155	94.9	78-127
Sodium***	155.2	141-184	157.8	139-190	148.0	141-155
Potassium***	4.1	3.2-5.2	4.5	3.5-5.9	4.4	2.9-6.6
CO2, Total***	29.6	16-44	29.0	14-41	28.3	21-35
BUN*	49.5	6-112	39.5	15.3-61	33.4	24.2-45.7
Creatinine*	0.3	0.2-0.7	<.2	<.2-.3	<.2	0
Total Protein*	7.5	5-11.2	3.9	2-5.8	4.4	3.4-5.2
Bilirubin, Total*	<.1	0	<.1	0	<.1	0
Calcium*	11.2	6.9-25.8	7.2	5.5-9.9	7.7	6.9-8.9
Phosphorus*	7.6	5.7-10.9	7.6	6.1-9.5	7.2	5.6-9.1
Uric Acid*	1.8	0.9-3.3	2.0	1.2-3.7	1.6	1.1-2
Alkaline Phosphatase**	45.7	24-82	49.4	25-87	61.9	39-90
ALT(SGPT)**	91.2	3-457	13.8	4-37	14.9	4-34
AST(SGOT)**	792.9	96-5647	306.8	146-453	299.4	189-434
LDH**	860.0	310-1751	647.2	371-1261	716.6	312-1747
CPK**	651.9	27-3266	663.3	73-1601	354.2	152-538
Amylase**	469.9	150-860	509.8	134-851	628.4	456-844
Lipase**	4.8	1-15	17.3	11-22	<10	0
GGT**	5.5	5-9	<5	0	<5	0
Cholesterol*	323.4	200-501	166.1	81-278	185.8	111-420
Triglycerides*	468.2	40-2575	100.5	31-189	178.3	65-451

* mG/dL
** U/L
*** MMOL/L

Table 1: Clinical chemistry values for healthy, diseased, and recovered turtles

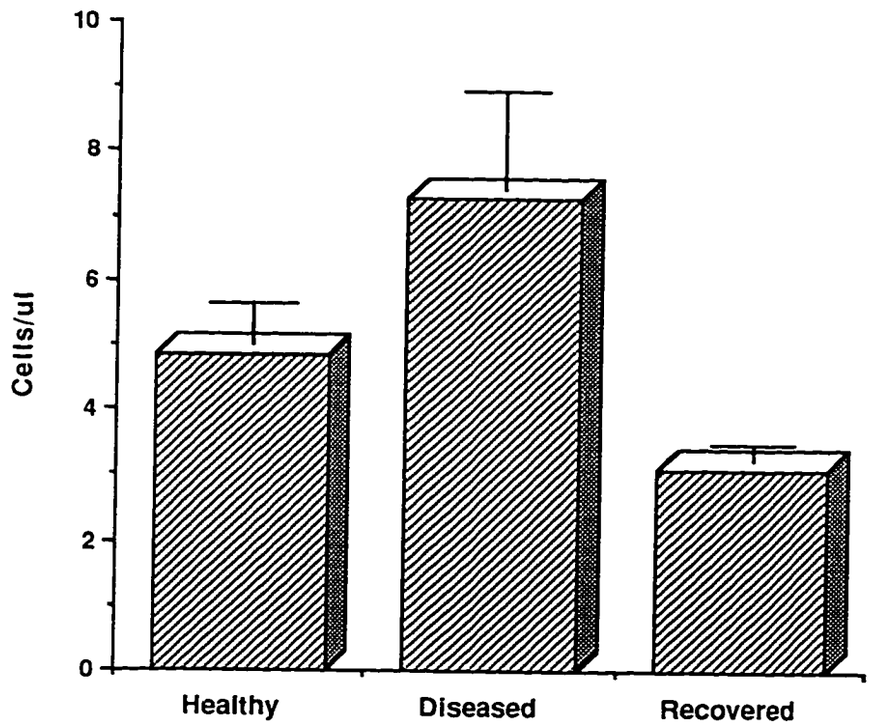

Figure 3: Total white blood cell counts for healthy, diseased, and recovered turtles

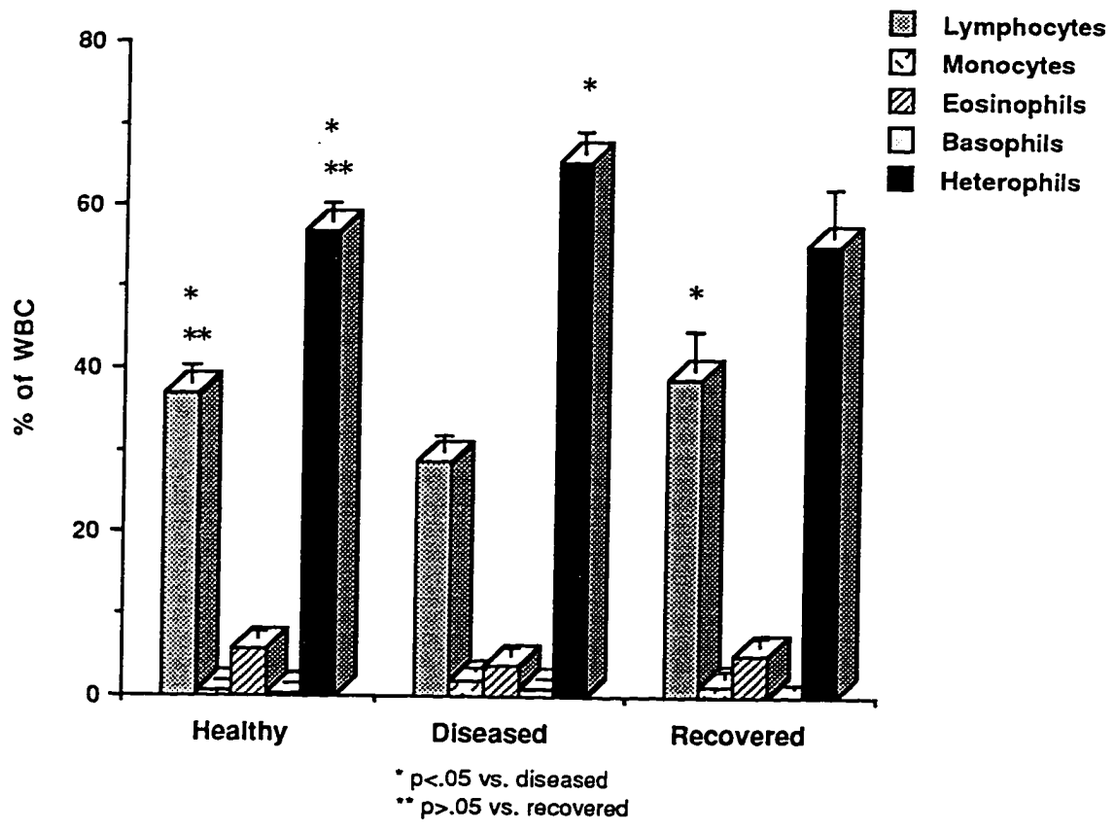

Figure 4: Leukocyte types and percentages for healthy, diseased, and recovered turtles

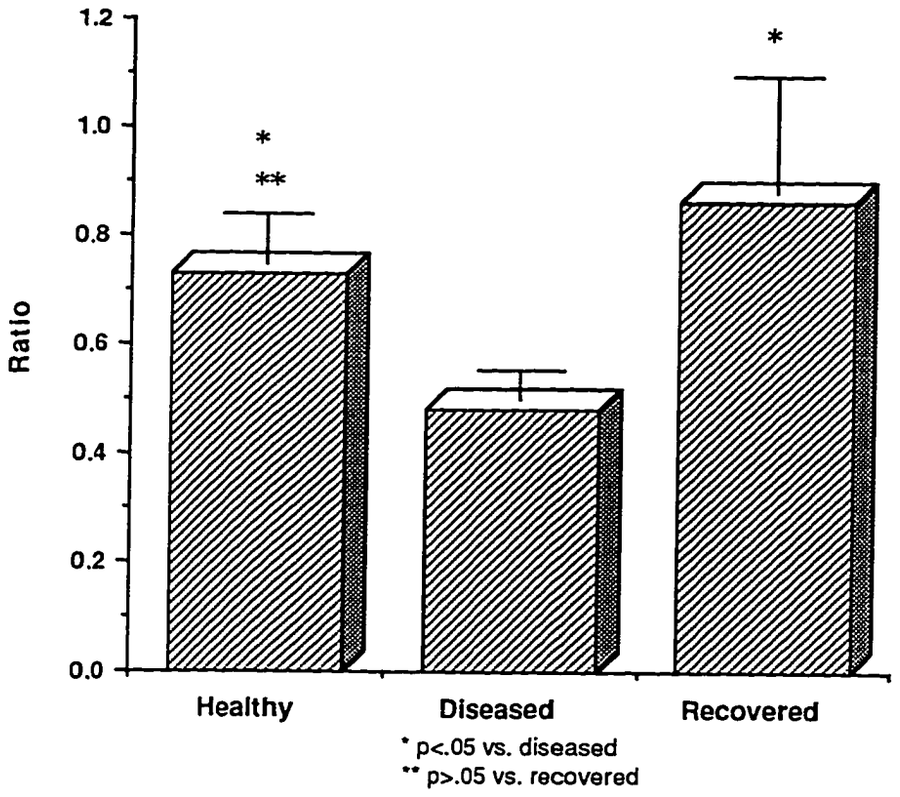

Figure 5: Lymphocyte/heterophil ratio for healthy, diseased, and recovered turtles

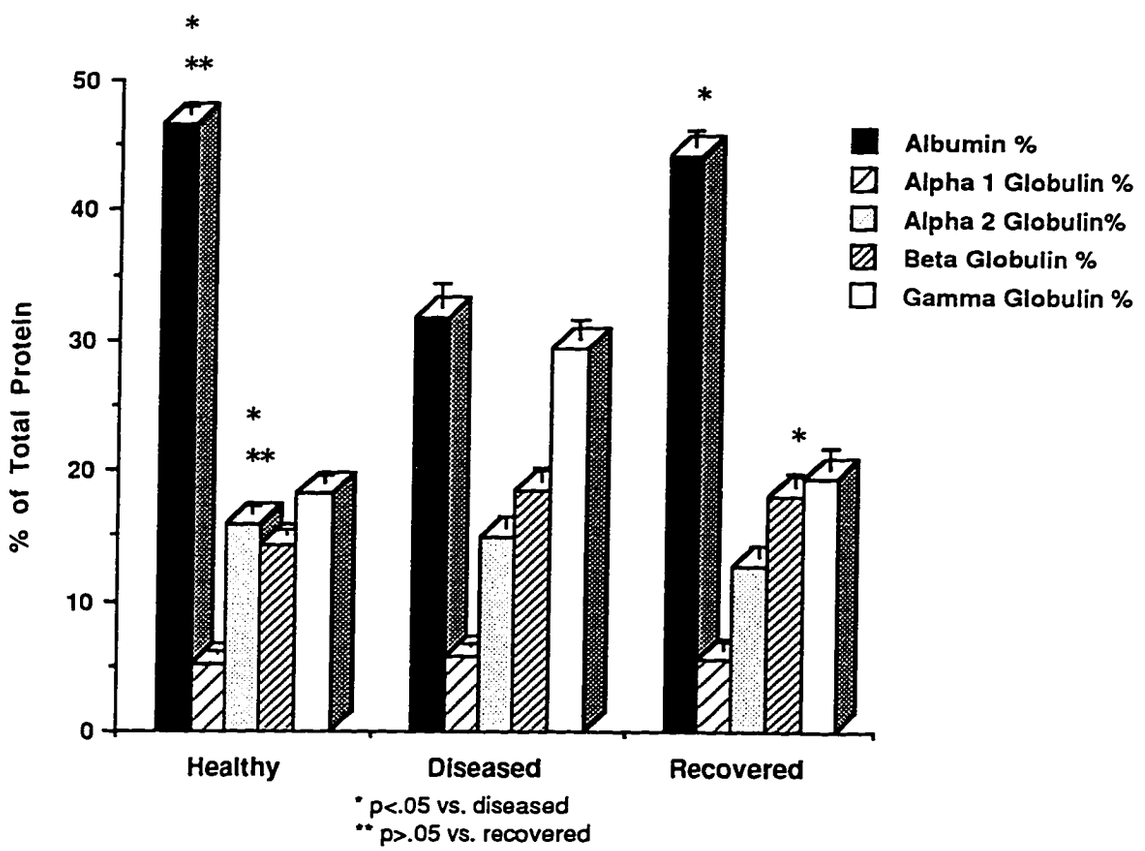

Figure 6: Serum proteins for healthy, diseased, and recovered turtles

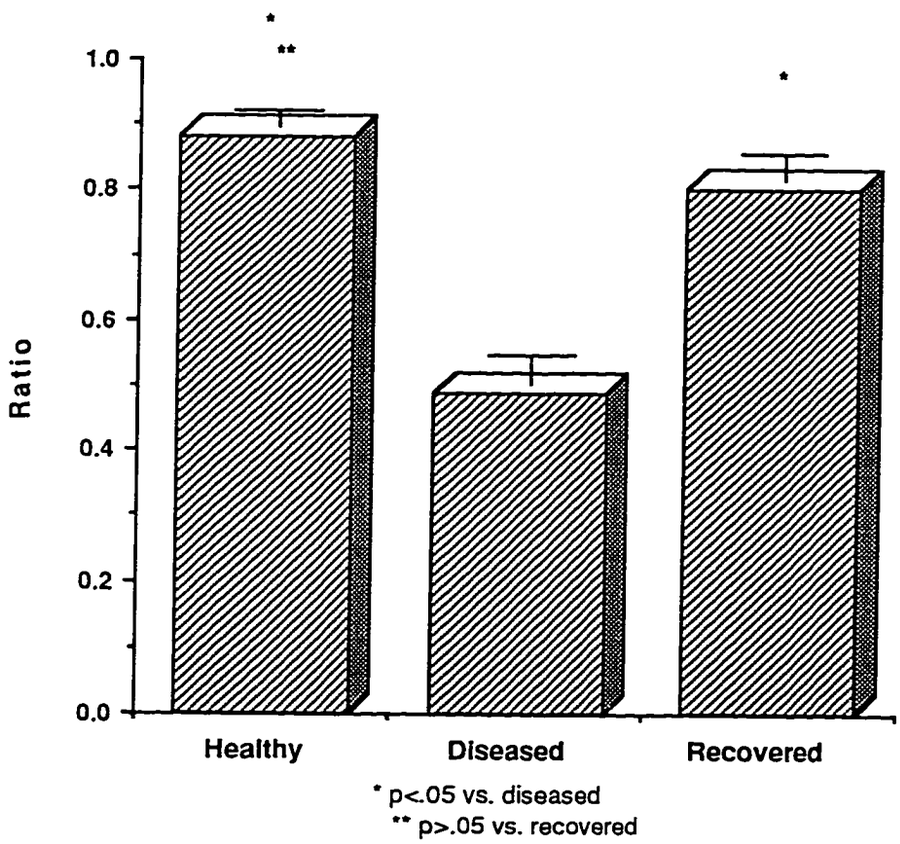

Figure 7: Albumin/globulin ratio for healthy, diseased and recovered turtles

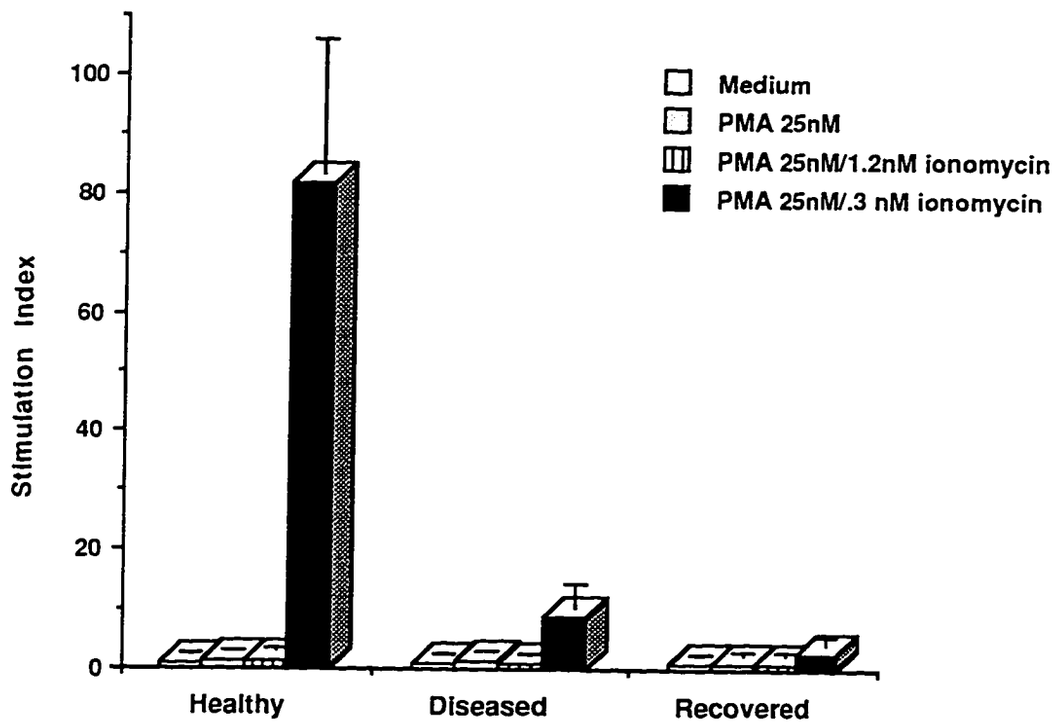

Figure 8: Stimulation with varying concentrations of PMA and Ionomycin

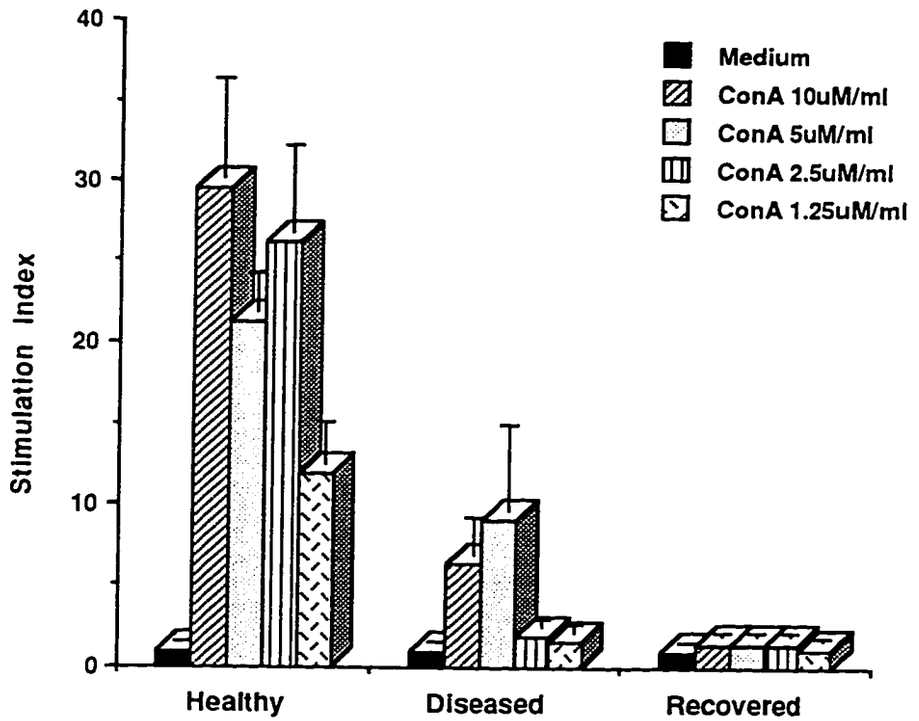

Figure 9: Stimulation with varying concentrations of ConA

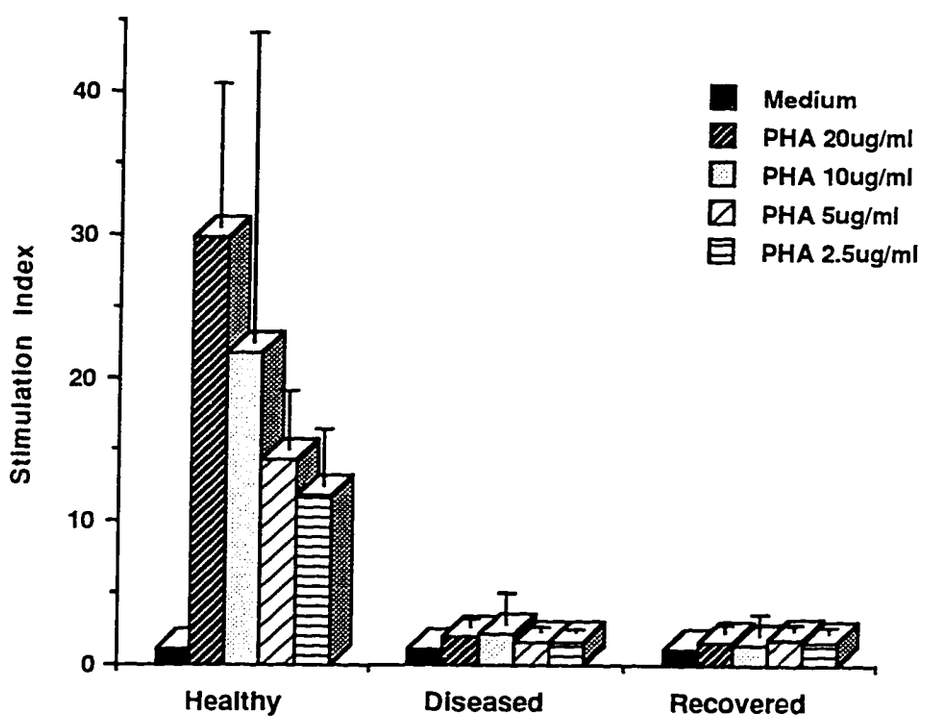

Figure 10: Stimulation with varying concentrations of PHA

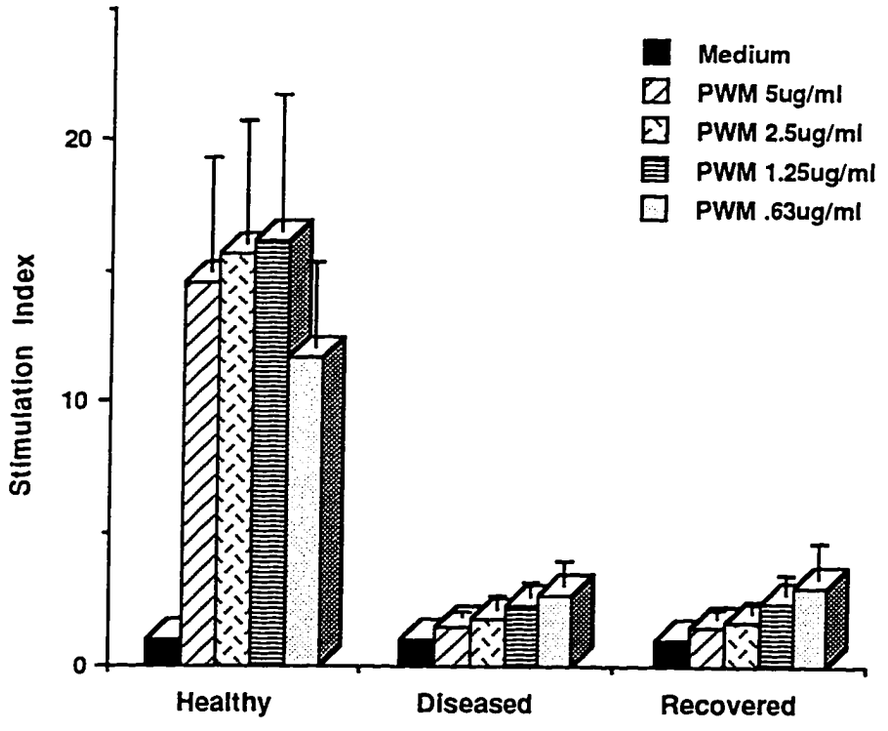

Figure 11: Stimulation with varying concentrations of PWM

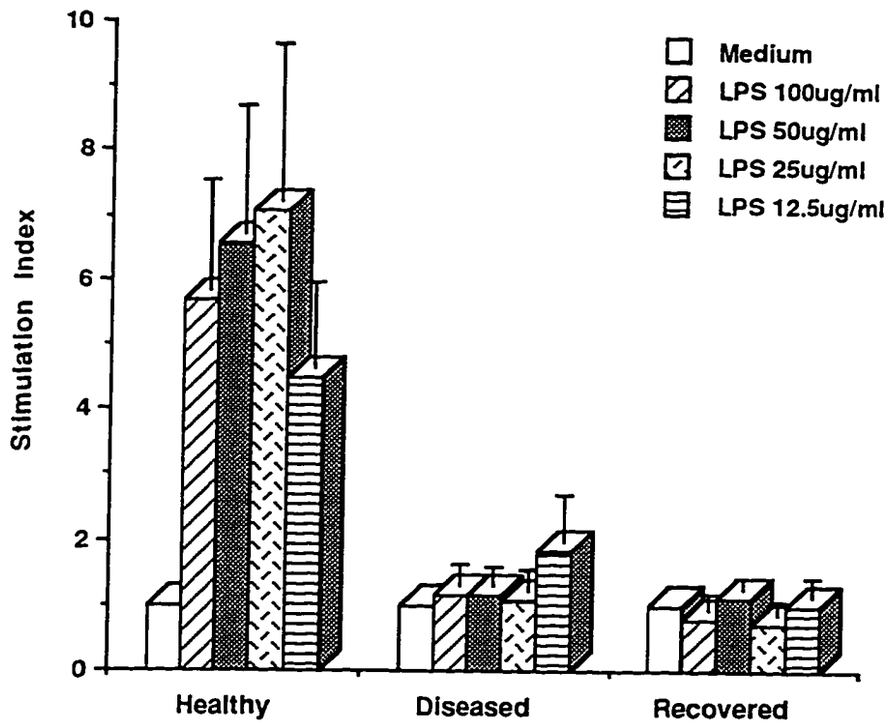

Figure 12: Stimulation with varying concentrations of LPS

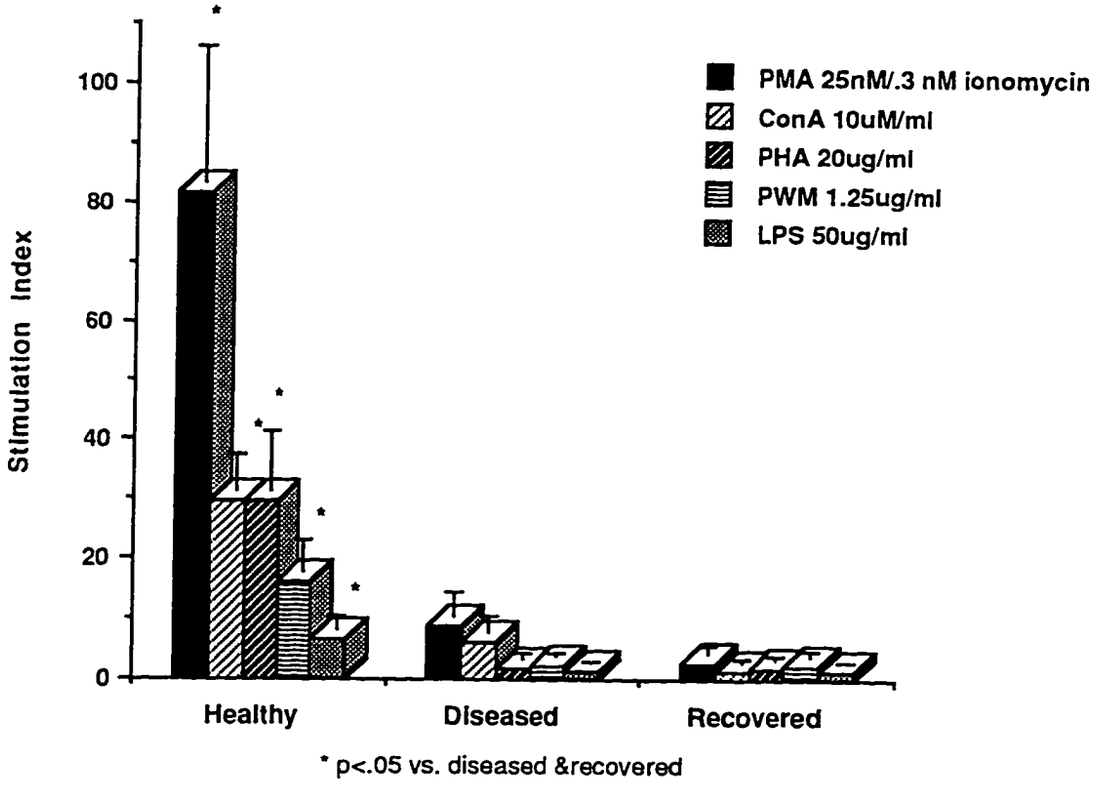

Figure 13: Best lymphocyte stimulation values for healthy, diseased, and recovered turtles

DISCUSSION

This investigation focused on procuring a clinical profile of green turtle fibropapillomatosis. There was every indication that clinical chemistry, complete blood count, serum protein electrophoresis, and *in vitro* lymphocyte stimulation assays would work on the green sea turtle, but little indication was given of their use in clinical diagnosis. For the purposes of this investigation each assay was analyzed on an individual basis and its applicability to this disease was determined.

Clinical chemistry

The healthy animals in this investigation displayed very broad ranges in nearly every chemistry value. This same phenomenon has been seen in wild caught loggerhead sea turtles (Lutz P & Dunbar-Cooper A, 1986). It was also seen that these broad ranges for the healthy animals actually encompassed those of the diseased animals. Thus the overlapping quality of these ranges actually hindered the values' use as standards from which to judge. Without being able to distinctly separate out a healthy, diseased, or recovered animal on the bases of these values, clinical chemistry is useless as diagnostic for this disease. The utilization of clinical chemistry would be more realistic for individual long-term residents with extensive histories of chemistry blood work.

Hematology

Several interesting aspects arose from the hematological work performed in this investigation. A small, but insignificant, rise in total white blood cells was noticed for diseased animals. This white blood cell count was composed of primarily lymphocytes and heterophils, in an interrelated fashion. Diseased animals showed a significantly decreased lymphocyte count, and significantly increased heterophil count when compared to both healthy and recovered turtles. Upon studying the lymphocyte/heterophil ratio, the same trend was noticed, indicating an inverse coupling between lymphocytes and heterophils that was conserved between the three groups. This increased heterophil count in diseased animals could most likely be attributed to stress, neoplasia, and nonspecific inflammatory responses. At the same time, decreased lymphocytes in diseased animals are most likely associated with stress. While the lymphocyte /heterophil ratio clearly drops for the diseased animals, a very definite increase in this ratio occurs in the recovered animals to the healthy group level. This gives every indication that not only is there cellular recovery in the recovered group, but that the level to which they are recovering is healthy by our "healthy" group standards. Their highly conserved values makes lymphocyte /heterophil ratio a good tool for indicating that the animal is under extreme stress conditions, and that some medical assistance in necessary.

Serum Protein Electrophoresis

Serum protein electrophoresis was employed in determining the active role of serum in the immune response of these animals. The two primary serum proteins proved to be albumin and gamma globulin, in an inversely related fashion. A significant decrease in albumin and significant increase in gamma globulin for the diseased group as compared to the healthy and recovered groups was noticed, with no difference noticed between the healthy and recovered groups for both proteins. This translated well to the albumin/globulin ratio where the same significant trends were noted. A decrease in albumin could most likely be associated with nutritional deficiency as a secondary factor to this disease. Increased gamma globulin, thus increased globulin, could simply be an albumin displacement phenomenon, where a decrease in the percentage of serum albumin would increase, by default, the weight of the globulin portion of the total serum protein. While some of this increased globulin may be attributed to mild humoral immune activity, it is unlikely that an immune response to so many possible secondary pathogens would amount to significant rise in only gamma globulin.

In vitro lymphocyte stimulation

This direct test of immune function for each of the test groups was conducted using several types of lymphocyte stimulants, each displaying a specificity of cell type stimulation. PMA in conjunction with ionomycin is known to be an overall B and T cell blastogenic stimulant, resulting in a higher index of stimulation when compared to subpopulation specific stimulants. The concentration of 25nM

PMA/.3nM ionomycin proved to work the best in all the groups, also having the highest overall index of proliferation overall.

Concanavalin A (ConA) has been documented as a specific T cell subpopulation blastogenic stimulant, having an index of proliferation second only to PMA/ionomycin, due to the weight of T cell presence in normal lymphocyte populations. A concentration of 10 μM/ml ConA proved to be the most effective in all the test groups, and its index of stimulation was second only to PMA/ ionomycin.

Phytohemagglutinin (PHA) is also a known T cell stimulant, with similar blastogenic qualities to ConA. Optimal performance for PHA was seen at 20μg/ml, with a stimulation index about the same as that of ConA.

Pokeweed mitogen (PWM) is known as a weak B and T cell blastogenic stimulant, with a variable response according to subject species. It proved a poor lymphocyte stimulant with sea turtles even at its optimal concentration of 1.25μg/ml, with a stimulation index lower than those of ConA and PHA.

The known B cell stimulant was lipopolysaccharide (LPS), which because of its isolated lymphocyte subpopulation influence and the relatively slow response of B cells, was expected to have the lowest index of proliferation overall. This investigation revealed a distinct B-like cell subpopulation, with the lowest overall index of stimulation in all groups when LPS was applied at its optimal concentration of 50μg/ml.

Several interesting points arose from this portion of the investigation. Firstly, a lymphocyte heterogeneity is evident in the

green sea turtle with distinct subpopulations responding appropriately to corresponding lymphocyte stimulants. It is also clear that healthy green sea turtle lymphocytes can respond quite vigorously to perceived pathogens. Most importantly, diseased and recovered green sea turtles show a significantly diminished response to pathogen.

In vitro lymphocyte proliferation has proven to be a most powerful tool for determining the true health of green sea turtles. Clinical chemistry, hematology, and serum protein electrophoresis masked the root problem inherent with GTFP, revealing symptoms in the diseased animals but indicating that the recovered group had truly recovered. While two dimensionally the recovered animals show appropriate improvements in lymphocyte/heterophil and albumin/globulin ratios, this evidence contradicts their apparent immune incompetence. It becomes feasible to believe that the leukocyte and specialized chemistry values may be misinterpreted if applied without this important immune background. Serum albumin percentage may increase after diet is improved in rehabilitation treatment. Lymphocyte counts may increase and heterophil counts decrease after a stress episode has passed during prolonged rehabilitation treatment. The crucial point to be considered is that even any new lymphocytes that may have been produced are showing a diminished ability to respond to antigenic stimulation. This indicates that a form of T lymphocyte dysfunction is the most likely diagnosis in these animals. This would make the true prognosis for

recovery dim, with these affected animals maintaining immune systems that are essentially inviable.

Conclusions

This investigation marks the first time that the immune system of the sea turtle has been described in detail. This new information has allowed for a great deal of knowledge to be gained regarding immune function in diseased green sea turtles. The data have indicated that animals afflicted with fibropapillomatosis demonstrate a serious compromise of their immune system. Furthermore, that "recovered" animals present a secondary immune compromise which can be linked to fibropapillomatosis as well. In forming a clinical pathology profile, clinical chemistry has proven to be useless but albumin/globulin and lymphocyte/heterophil ratios have proven to be useful tools for general health assessments in these animals. It is clear however that further work is necessary in ascertaining the root of this compromise.

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