SOUTHWEST FISHERIES CENTER

NATIONAL MARINE FISHERIES SERVICE HONOLULU LABORATORY 2570 DOLE STREET HONOLULU, HAWAII 96822-2396

JULY 1996

# MICROSCOPIC AND ULTRASTRUCTURAL EVIDENCE OF A HERPESVIRUS-LIKE VIRUS IN HAWAIIAN GREEN TURTLES (*CHELONIA MYDAS*) WITH FIBROPAPILLOMATOSIS

A. ALONSO AGUIRRE
AND
TERRY R. SPRAKER

Honolulu Laboratory
Southwest Fisheries Science Center
National Marine Fisheries Service, NOAA
Honolulu, Hawaii 96822-2396

ADMINISTRATIVE REPORT H-96-06C

Southwest Fisheries Science Center
Administrative Report H-96-06C



# MICROSCOPIC AND ULTRASTRUCTURAL EVIDENCE OF A HERPESVIRUS-LIKE VIRUS IN HAWAIIAN GREEN TURTLES (*CHELONIA MYDAS*) WITH FIBROPAPILLOMATOSIS



A. Alonso Aguirre[1] and Terry R. Spraker[2]

Honolulu Laboratory
Southwest Fisheries Science Center
National Marine Fisheries Service, NOAA
2570 Dole Street, Honolulu, Hawaii 96822-2396

[1]Colorado State University, P.O. Box 1522, Fort Collins, Colorado 80522

[2]Wildlife Pathology International, 2905 Stanford Road, Fort Collins, Colorado 80525

July 1996

NOT FOR PUBLICATION

# PREFACE

This report by Drs. A. Alonso Aguirre and Terry R. Spraker presents the results of research in which evidence was found of a herpesvirus-like virus present in fibropapilloma tumors of the Hawaiian green turtle, *Chelonia mydas*. The work was conducted with funds awarded by the Southwest Fisheries Science Center (SWFSC) Honolulu Laboratory's Marine Turtle Research Program. The results of previous research conducted under contracts awarded to Dr. Aguirre can be found in SWFSC Administrative Reports H-92-07C, H-93-07C, H-93-11C, H-94-4C, H-94-09C, and H-95-01C issued by the Honolulu Laboratory.

The incidence of life-threatening tumors on green turtles in the Hawaiian Islands has grown to epidemic proportions during the past decade. A similar situation exists among green turtles at certain sites in Florida, the Caribbean, and elsewhere worldwide. The cause of this disease, called fibropapillomatosis, remains unknown. However, a herpes virus has been strongly implicated in studies conducted with green turtles in Florida. Death appears to be the usual result of the disease, although the impact to afflicted populations has not been fully assessed. The disease represents one more potentially significant threat to the survival of all green turtles. Recent finding in Florida have shown that the disease is now also occurring in increasing numbers in the loggerhead turtle, *Caretta caretta*.

The nature of fibropapillomatosis, along with its exact cause and mode of transmission, must be determined in order to develop a long-term management program of containment and prevention. The findings of the present report constitute progress in this direction which must be followed by additional research.

Because this report was prepared by independent investigators, its statements, findings, conclusions, and recommendations do not necessarily reflect the views of the National Marine Fisheries Service, NOAA.

George H. Balazs
Leader, Marine Turtle Research
July 1996

Honolulu Laboratory
Southwest Fisheries Science Center
National Marine Fisheries Service, NOAA
2570 Dole Street
Honolulu, Hawaii 96822-2396

## ABSTRACT

This study reports the histopathologic (17) and ultrastructural (9) examination of biopsies collected from early fibropapillomas (< 2 mm to 2 cm) found in green turtles (*Chelonia mydas*) collected at Kaneohe Bay, Oahu, and Palaau, Molokai, in the Hawaiian Islands. In addition, marine leeches, *Ozobranchus branchiatus*, attached to extremely small fibropapillomas (< 2 mm) found in the cantus of the eyes were examined under light and electron microscopy (EM) for the presence of haemogregarines, herpesvirus-like particles, inclusion bodies, or other structures that could resemble agents of an infectious nature. Histopathologic description of fibropapillomas was similar to reports previously published for the Hawaiian Islands and Florida. Two of 17 tumor biopsies from different turtles yielded the presence of intranuclear inclusion bodies. In addition, intranuclear inclusion bodies within keratinocytes of one of nine biopsies observed under EM, yielded the presence of a herpesvirus-like virus in various stages of replication. No ultrastructural evidence was identified to implicate marine leeches as possible mechanical or biological vectors in the transmission of these herpesvirus-like particles or other infectious agents related to fibropapillomas in the specimens analyzed. The histopathologic findings and the virus seen by EM supported a viral etiology with spirorchid trematode eggs and their associated granulomata being secondary to fibropapilloma development. Further research should emphasize the isolation and characterization of this agent to provide possible insights and solutions in the control, treatment, and prevention of this disfiguring and debilitating disease.

## INTRODUCTION

Green turtle fibropapillomatosis (FP) affects green turtle (*Chelonia mydas*) populations in epizootic proportions throughout the world (Williams et al., 1994). Recent research findings support that viral particles similar to those of a herpesvirus-like virus may act as the etiologic agent (Herbst et al., 1995); however, this virus has not been isolated or characterized. Other etiologies have been suggested including other viruses i.e., papillomavirus, retrovirus, poxvirus, and spirorchid trematodes and their ova (Balazs and Pooley, 1991; Dailey and Morris, 1994).

The conspicuous infestation of fibropapillomas with marine leeches (*Ozobranchus branchiatus*) continues to raise the question of their role as possible vectors of the FP primary etiologic agent. First reported by Nigrelli (1942) attached to fibropapillomas of green turtles, marine leeches were suggested as possible vectors of FP (Nigrelli and Smith, 1943). These piscicolid metazoans are known to transmit haemogregarines and may cause increased vascularization on their site of attachment by secreting hirudin; in addition, they may cause severe debilitation of the host during massive infestations (Schwartz, 1974; Lauckner, 1985).

The objective of this study was to evaluate normal skin and small FP growths by histopathologic examination and electron microscopy and attempt to find and identify the herpesvirus-like particles previously described in other studies (Jacobson et al., 1991; Herbst et al., 1995). In addition, the ultrastructure of marine leeches was examined to identify their possible role as vectors of the FP primary etiologic agent.

## MATERIALS AND METHODS

Biopsies were collected in the Hawaiian islands from clinically healthy green turtles and turtles with FP, at Palaau (21°06'N, 157°07'W) along the south shore, Island of Molokai, during July 1995, and in Kaneohe Bay (21°30'N, 157°50'W), Island of Oahu, during October 1995. Green turtles at Molokai were captured live and unharmed using a nonentangling impoundment net. Turtles in Kaneohe Bay were captured by hand while snorkeling from a boat. Turtles were sampled for blood, measured, tagged, and weighed (Owens and Ruiz, 1980; Balazs et al., 1987). All turtles were thoroughly examined for the presence of fibropapillomas and a description of their size, number, and location was recorded when available. Turtles were coded by a degree of fibropapilloma severity score on a scale of 0-4, with a score code = 4 being the most severe case. The approximate scale categories for individual tumors included code 1 = detectable

patch to 1 cm diameter; code 2 = >1 cm to 4 cm; code 3 = >4 cm to 10 cm; and code 4 = > 10 cm. Anatomic site influenced degree of severity rating when vision or ability to feed was impaired (Balazs, 1991).

Following topical or subcutaneous infiltration of a local anesthetic (2% lidocaine) multiple tissue biopsies were collected from the normal skin and fibropapillomas of neck, head, and flippers paying special attention to tissues surrounding the canthus of both eyes and in the oral cavity. Field observations in Hawaii have indicated that primary infection and evidence of FP is often first visible in the canthus of either eye or in the mouth. Tissues were collected with a disposable 6-mm Baker's dermal punch® (Webster, Inc., Sterling, Massachusetts, USA) or by excisional biopsy. Individual fibropapillomas measuring <1 mm to 20 mm were collected and analyzed. Pigmentation, surface, attachment, and location of growths were recorded. Normal skin biopsies taken from the inguinal regions of clinically healthy turtles served as controls. Marine leeches were collected when attached to early growths (up to 2 mm) in the canthus of the eye and in the temporomandibular joints. Biopsies and leech specimens were split into two vials. One vial contained 10% neutral buffered formalin for histopathologic evaluation. A second vial contained Karnovsky's solution for electron microscopic evaluation. Both vials were held at 4°C until processed.

In the laboratory, skin biopsies and fibropapilloma lesions were embedded in paraffin, sectioned 6-$\mu$m thick and stained with hematoxylin and eosin and GMS (Gomori methenamine-silver nitrate stain) for the identification of fungi. For electron microscopy, tissues and marine leeches were washed with 0.2 M Sorenson's phosphate buffer pH 7.3 and were postfixed in 1.0% osmium tetroxide for 1 h. Specimens were washed through two changes of $ddH_2O$, dehydrated through a graded acetone series, infiltrated with and embedded in Medcast-Araldite 502 Resin® (Ted Pella Inc., Redding, CA) (Hayat, 1986). Ultrathin sections from two skin biopsies, eight FP biopsies and two leeches were placed on copper grids, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope by Dr. John Chandler (Electron Microscopy Center, Department of Anatomy and Neurobiology, Colorado State University, Fort Collins, CO 80523).

## RESULTS

Nine clinically healthy green turtles collected in Kaneohe Bay provided the control skin biopsies obtained from the right or left hind flipper towards the inguinal area. These turtles had a mean (±SD) straight carapace length (SCL) of 44.5 (±3.6) cm (range 40.4-49.6 cm) and a mean (±SD) weight of 13.6 (±3.5) kg (range 9.1-17.2 kg).

Eight green turtles sampled at Palaau, Molokai, with a mean (±SD) SCL of 60.1 (±6.0) cm (range 46.6-67.2 cm) with overall fibropapilloma code severity = 2 (range 1-3); and nine green turtles sampled at Kaneohe Bay, Oahu, with a mean (±SD) SCL of 54.9 (±7.7) cm (range 40.9-67.2 cm) and a mean (±SD) weight of 25.1 (±9.6) kg (range 10.4-43.1 kg) with overall FPS = 2 (range 1-3) provided fibropapilloma biopsies for light and electron microscopic examination.

Multiple sections of nine skin biopsies were analyzed under light microscopy. In all biopsies the normal skin contained a few vessels within the dermis that were cuffed with lymphocytes, plasma cells, and an excessive amount of pigment. In most cases, the thickness of the epidermis was relatively uniform composed by four to eight cell layers and within normal limits. The keratin layer was thin and uniform. The dermis presented a papillary layer and a well defined reticular layer composed of large bundles of collagen. Dermal papillary projections into the epidermis were also observed (Fig. 1).

Focal dermatitis within a lymphocytic epidermis was observed in 56% (5/9) of the biopsies. These foci were characterized by mild acanthosis and early necrosis within the dermis. Although not visible at gross examination in the field, there were marine leeches attached to the skin of four of nine biopsies. The areas of leech attachment were ulcerated, shallow, and surrounded by inflammatory cells.

Fibropapillomas ranging from 1 x 1 mm to 10 x 12 mm, were characterized as small, papillary, early growths within the epidermis and dermis. Their epidermal epithelium was acanthotic and presented early pseudoepitheliomatous hyperplasia covering a proliferative layer of fibroblastic cells, with a reactive superficial dermal mesoderm. In 41% (7/17) of the biopsies taken, the tumors were plaque-like (flat sarcoid) in shape and tightly adherent to the underlying epidermis (Fig. 2).

Fibropapillomas initially proliferated at the basal layer of epidermis and then progressed downward structurally forming folds and papillary projections. The core of the papillary projections was composed of fusiform cells surrounded by finely fibrillar cytoplasm. Ballooning degeneration and nuclear necrosis were evident in keratinocytes (Fig. 3). Areas of ulceration on the surface of four FP biopsies extended into the superficial dermal tissue. Ulcers in the epidermis of two FP biopsies were characterized by swelling and hypertrophic degeneration of the epithelium with structures similar to intranuclear inclusion bodies. The inclusions were characterized by large basophilic structures within the nucleus, some of which occupied the entire nucleus producing margination of chromatin. Vessels surrounding these areas presented lymphocytic infiltration and were cuffed with plasma cells. Hyperplasia of the basal cell layer of the epidermis was observed with a reactive upper dermis characterized

by proliferation of fusiform or fibroblastic cells. Spirorchid trematode eggs were identified in 41% (7/17) of FP biopsies. A granulomatous reaction was associated with these spirorchid trematode ova. These granulomas were observed within the fibropapilloma and within the connective tissue adjacent to the growth (Fig. 4). Other organisms were present on the surface of 10/17 (59%) fibropapillomas including fungi (5/17), bacteria (4/17), leeches (2/17), mites (1/17), and algae (1/17) (Fig. 5). Occasionally, keratinocytes presented vacuole degeneration, blister formation, and pigmented macrophages.

Electron microscopic findings of nine biopsy specimens included multiple epidermal folds associated with dermal proliferation and epidermis thicker than normal. Dermal papillae were projected towards the epidermis, and intercellular spaces were enlarged. Increased numbers of subcellular organelles, endoplasmic reticulum, mitochondria, and hypertrophy and hyperplasia of the stratum spinosum with pleomorphic cells were also identified.

Virus particles with electron-dense bodies measuring 100 to 110 mm in diameter were observed in the nuclei of keratinocytes derived from a pigmented, pedunculated papilloma measuring 5 x 9 mm collected from the neck of a turtle in Kaneohe Bay. Particles in different stages of development including empty capsids and icosahedral nucleocapsids were observed. One viral particle was identified as acquiring an envelope by budding through the nuclear membrane. These structures resembled viruses of the family Herpesviridae based on size and morphology (Fig. 6).

Electron microscopic analysis for the presence of similar structures, haemosporidia, or other infectious agents in two marine leeches yielded negative results.

## DISCUSSION

The histopathology of green turtle fibropapillomatosis has been previously described for green turtles in Florida (Jacobson et al., 1989) and Hawaii (Aguirre et al., 1994). Early lesions demonstrated characteristics of fibropapillomas as described for other species (Sundberg, 1992).

Inclusion bodies and herpesvirus-like particles found in a fibropapilloma biopsy correlate to results recently reported for green turtles in Florida (Jacobson et al., 1991; Herbst et al., 1995). The early ballooning degeneration, the intranuclear structures, and the nuclear necrosis of the epithelium were lesions highly suggestive of a viral infection of the epidermis, extending to the underlying dermis. Herpesvirus and herpesvirus-like infections have been reported in maricultured and wild green turtles as grey-patch disease of hatchlings (Rebell et al., 1975), pneumonia (Jacobson et al., 1986), and cutaneous

fibropapillomas (Jacobson et al., 1991). More recently Herbst et al. (1995) demonstrated that the etiologic agent of FP is an infectious filterable subcellular agent. This agent resembled a virus of the family Herpesviridae.

The present study identified a similar virus in one of nine fibropapilloma biopsies analyzed with an electron microscope. Viruses of the family Herpesviridae are known to induce neoplasia in lymphoid tissues of several species, ranging from amphibians and reptiles to birds and mammals (Fenner et al., 1987). Herpes viruses may act, however, as opportunistic or secondary agents to a primary process or infection caused by other viruses; i.e., papillomavirus, poxvirus, and retrovirus. Attempts to culture, identify, and characterize these virus-like particles have thus far been unsuccessful (Jacobson et al., 1991; Herbst et al., 1995).

Intranuclear inclusion bodies were identified in only a few keratinocytes (11.7%) of biopsies analyzed. And, although virus particles identified in this study were present in higher percentage (11%) of fibropapillomas than other reported studies in wild turtles (1.9%) (Herbst, 1995), many growths need to be examined to determine presence of inclusions and viral particles as suggested by Jacobson et al. (1991).

Other agents identified within the neoplastic tissue including spirorchid trematode ova--resulting in granulomata, mites, bacteria, fungi, and algae were considered secondary to tumor development. Secondary bacterial infections were observed causing ulcerated areas of the epidermis, whereas in other areas the primary infection produced epithelionecrosis and ulceration followed by bacterial proliferation. The present study did not provide evidence that spirorchid trematode eggs were the initial cause of fibropapilloma formation. The lesions observed did not represent a host response characterized by a cutaneous, foreign body fibrosis associated with papillary epidermal hyperplasia as previously hypothesized (Harshbarger, 1991; Dailey and Morris, 1995).

Several skin biopsies used as controls presented a mild to moderate dermatitis caused by marine leeches. These areas of ulceration and irritation have lead to the hypothesis that these parasites can act as vectors or increase the susceptibility of green turtles to skin penetration by an infectious agent. Prevalence and degree of infestation with these leeches is high in Hawaiian green turtles. During this study, on gross examination, 41% of turtles with fibropapillomas were infested with leeches and 44% of skin biopsies taken from clinically healthy turtles were confirmed histologically with evidence of leech infestation. The impact of these parasites may be severe for turtles with and without FP. These leeches complete their entire life cycle in chelonians and are known to cause severe epizootics of hirudiniasis in captivity (Schwartz, 1974;

Lauckner, 1985). In addition, *Ozobranchus* spp. may act as vectors of trypanosomes and haemogregarines in fresh water turtles. No blood parasites were identified in a survey of sea turtles and their leeches (Davies and Chapman, 1974); however, intracytoplasmic inclusion bodies have been reported recently for Hawaiian *C. mydas* (Aguirre, 1993). Further ultrastructural investigations are necessary to elucidate the nature of these inclusions and the possible role of marine leeches in their transmission.

In conclusion, this research supports earlier findings suggesting that cellular reaction and fibropapilloma formation is associated with a herpesvirus-like agent of an infectious nature. Further studies including culture and characterization of this virus, immunohistochemical localization of its antigen, or detection via gene probes will provide possible insights for the epidemiology, control, treatment, and prevention of this disfiguring and debilitating disease.

## ACKNOWLEDGMENTS

We gratefully acknowledge the logistic and field support of D. M. Ellis, S. K. K. Murakawa, and B. Zimmerman. R. Morris from Makai Animal Clinic, Kailua, Hawaii, is also acknowledged for his support. We are indebted to J. Chandler, Department of Anatomy, Colorado State University, for the ultrastructural identification of the virus. Previous drafts of this manuscript were reviewed by B. Zimmerman. This research was sponsored in part under contract No. 40ABNF502008 from the NMFS Southwest Fisheries Science Center, Honolulu, Hawaii.

## LITERATURE CITED

Aguirre, A. A.
1993. Inclusion bodies in red blood cells of Hawaiian green turtles (*Chelonia mydas*). Honolulu Lab., Southwest Fish. Sci. Cent., Natl. Mar. Fish. Serv., NOAA, Honolulu, HI 96822-2396. Southwest Fish. Sci. Cent. Admin. Rep. H-93-11C, 10 p.

Aguirre, A. A., G. H. Balazs, B. Zimmerman, and T. R. Spraker.
1994. Evaluation of Hawaiian green turtles (*Chelonia mydas*) for potential pathogens associated with fibropapillomas. J. Wildl. Dis. 30:8-15.

Balazs, G. H.
1991. Current status of fibropapillomas in the Hawaiian green turtle, *Chelonia mydas*. *In*: G. H. Balazs and S. G. Pooley (eds.). Research Plan for Marine Turtle Fibropapilloma. U.S. Dep. Commer., NOAA Tech. Memo., NOAA-TM-NMFS-SWFSC-156, p. 47-57.

Balazs, G. H. and S. G. Pooley.
1991. Research Plan for Marine Turtle Fibropapilloma. U.S. Dep. Commer., NOAA Tech. Memo., NOAA-TM-NMFS-SWFSC-156, 113 p.

Balazs, G. H., R. G. Forsyth, and A. K. H. Kam.
1987. Preliminary assessment of habitat utilization by Hawaiian green turtles in their resident foraging pastures. U.S. Dep. Commer., NOAA Tech. Memo., NOAA-TM-NMFS-SWFSC-071, 107 p.

Davies, R. W.
1978. The morphology of *Ozobranchus margoi* (Apathy) (Hirudinoidea), a parasite of marine turtles. J. Parasitol. 64:1092-1096.

Davies, R. W. and C. G. Chapman.
1974. First record from North America of the piscicolid leech, *Ozobranchus margoi*, a parasite of marine turtles. J. Fish. Res. Board Can. 31:104-106.

Fenner, F., P. A. Bachmann, E. P. J. Gibbs, F. A. Murphy, M. J. Studdert, and D. O. White.
1987. Veterinary virology. Academic Press, San Diego, California, 660 pp.

Harshbarger, J. C.
1991. Sea turtle fibropapilloma cases in the Registry of Tumors in Lower Animals. *In*: Balazs, G. H. and S. G. Pooley (eds.). Research Plan for Marine Turtle Fibropapilloma. U.S. Dep. Commer., NOAA Tech. Memo., NOAA-TM-NMFS-SWFSC-156, p. 63-70.

Hayat, M. A.
1986. Basic techniques for transmission electron microscopy. Academic Press, Florida, pp. 226-227.

Herbst, L. H.
1995. The etiology and pathogenesis of green turtle fibropapillomatosis. Ph.D. Dissertation. University of Florida, Gainesville, Florida, 284 pp.

Herbst, L. H., E. R. Jacobson, R. Moretti, T. Brown, J. P. Sundberg, and P. A. Klein.
1995. Experimental transmission of green turtle fibropapillomatosis using cell-free tumor extracts. Dis. Aquat. Org. 22:1-12.

Jacobson, E. R., C. Buergelt, B. Williams, and R. K. Harris.
1991. Herpesvirus in cutaneous fibropapillomas of the green turtle *Chelonia mydas*. Dis. Aquat. Org. 12:1-6.

Jacobson, E. R., J. L. Mansell, J. P. Sundberg, L. Hajjar, M. E. Reichmann, L. M. Ehrhart, M. Walsh and F. Murru.
1989. Cutaneous fibropapillomas of green turtles (*Chelonia mydas*). J. Comp. Pathol. 101:39-52.

Jacobson, E. R., J. M. Gaskin, M. Roelke, E. Greiner, and J. Allen.
1986. Conjunctivitis, tracheitis, and pneumonia associated with herpes virus infection in green sea turtles. J. Am. Vet. Med. Assoc. 189:1020-1023.

Rebell, G., A. Rywlin, and H. Haines.
1975. A herpesvirus-type agent associated with skin lesions of green turtles in aquaculture. Am. J. Vet. Res. 36:1221-1224.

Williams Jr., E. H., Bunkley-Williams, L., Peters, E. C., Pinto-Rodriguez, B., Matos-Morales, R., Mignucci-Giannoni, A. A., Hall, K. V., Rueda-Almonacid, J. V., Sybesma, J. V., Bonnelly de Calventi, I. and Boulon, R. H.
1994. An epizootic of cutaneous fibropapillomas in green turtles *Chelonia mydas* of the Caribbean: part of a panzootic? J. Aquat. Anim. Health 6:70-78.

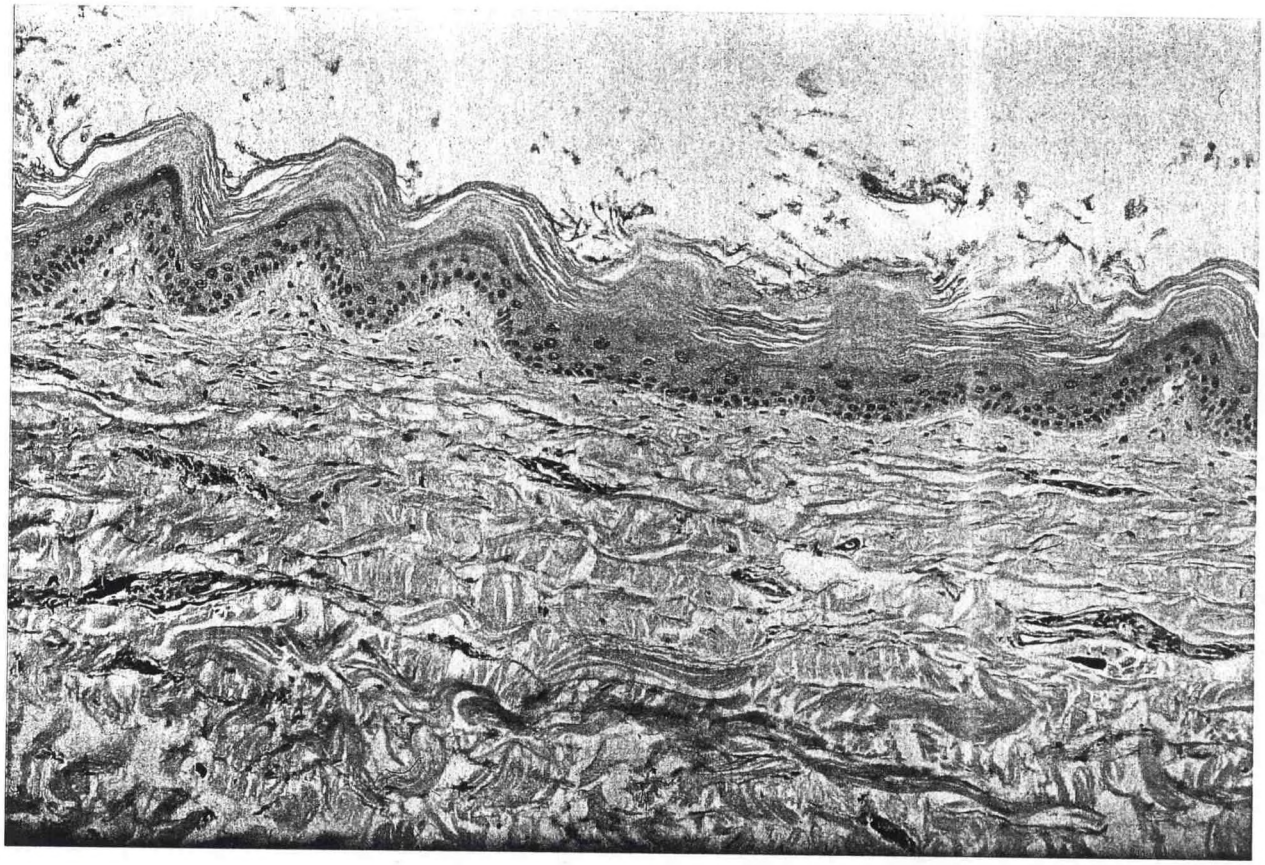

Figure 1.--Normal skin of a green turtle (*Chelonia mydas*), Kaneohe Bay, Island of Oahu, Hawaii, 1995. HE X 10.

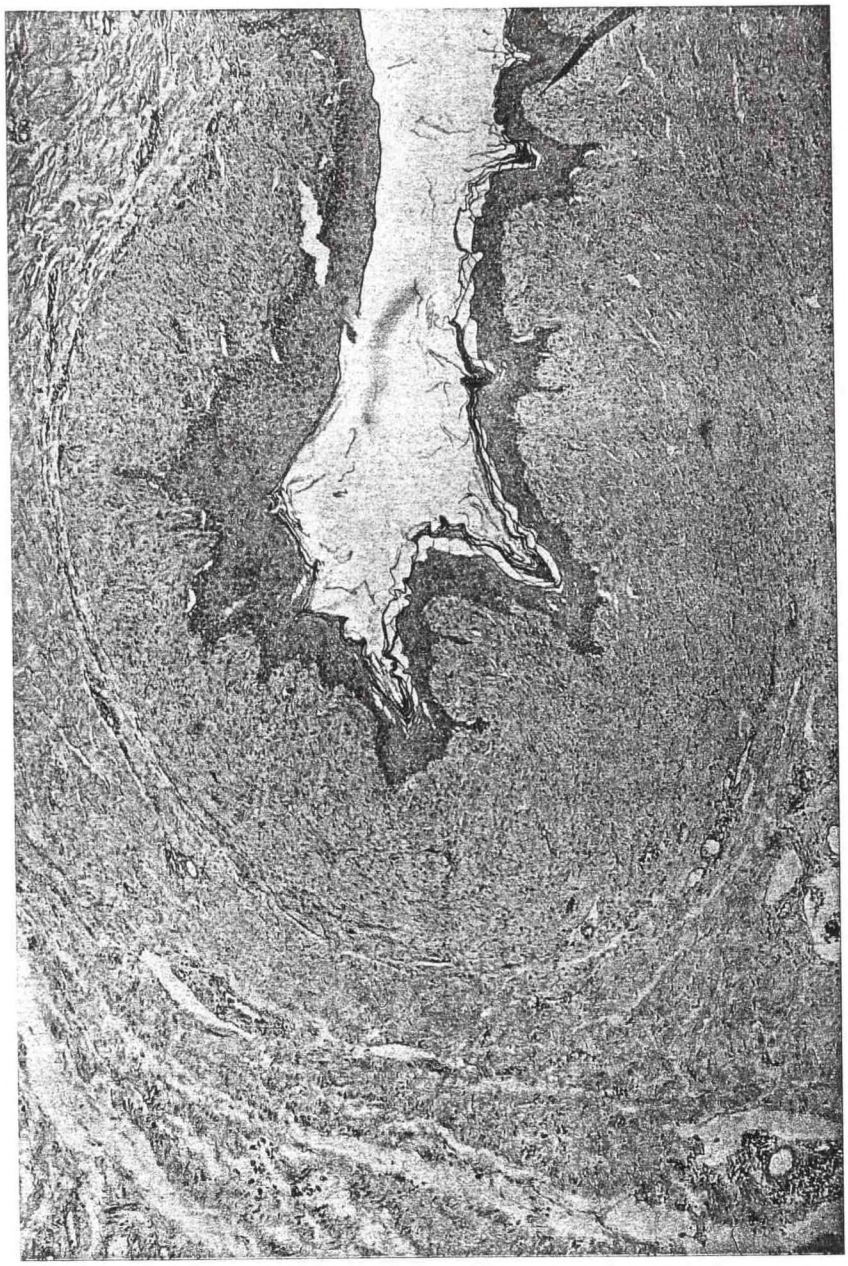

Figure 2.--Flat sarcoid in an early growth within the dermis with early pseudoepitheliomatous hyperplasia and acanthosis of the epidermis of a green turtle (*Chelonia mydas*), Kaneohe Bay, Island of Oahu, Hawaii, 1995. HE X 4.

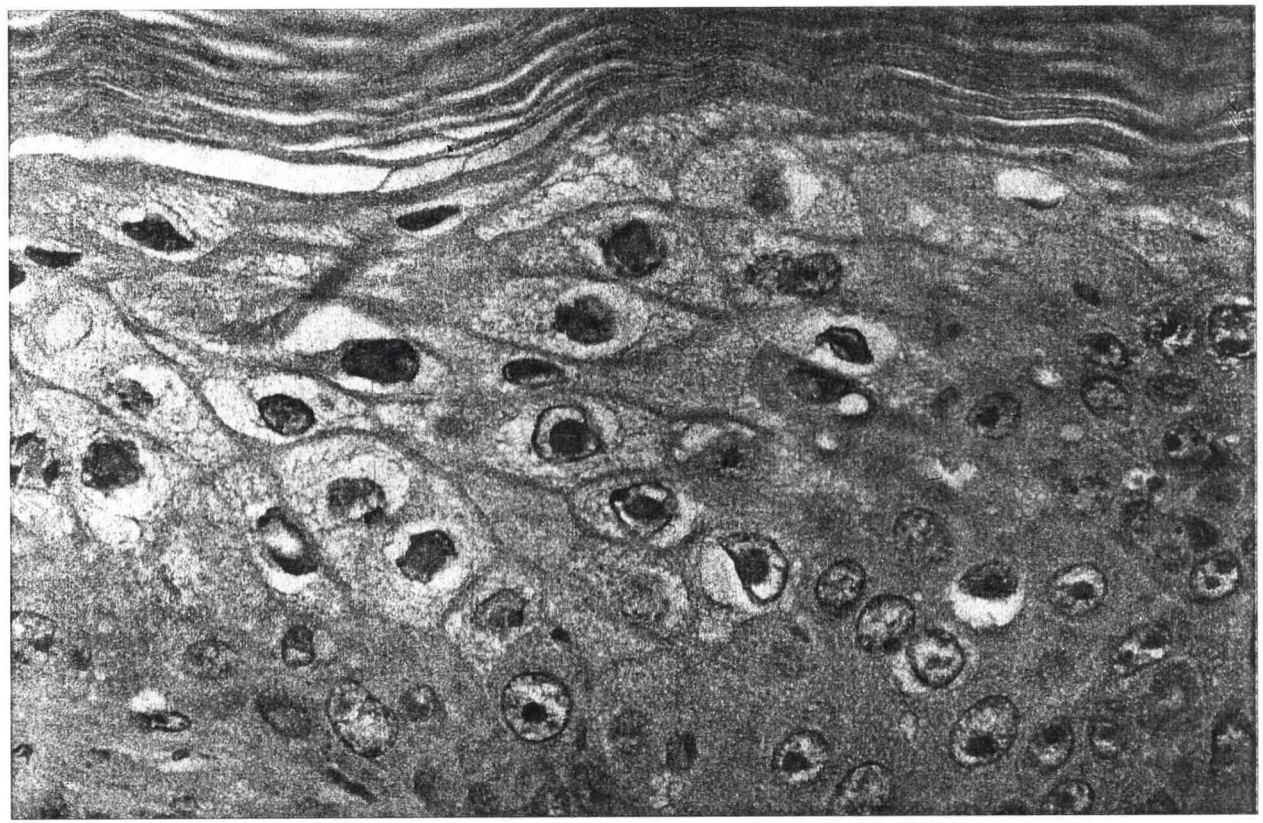

Figure 3.--Early ballooning degeneration, inflammatory reaction, and nuclear necrosis of stratum basale of epidermis in a fibropapilloma of a green turtle (*Chelonia mydas*), Kaneohe Bay, Island of Oahu, Hawaii, 1995. HE X 40.

Figure 4.--Spirorchid trematode granuloma in the fibropapilloma of a green turtle (*Chelonia mydas*), Kaneohe Bay, Island of Oahu, Hawaii, 1995. HE X 4.

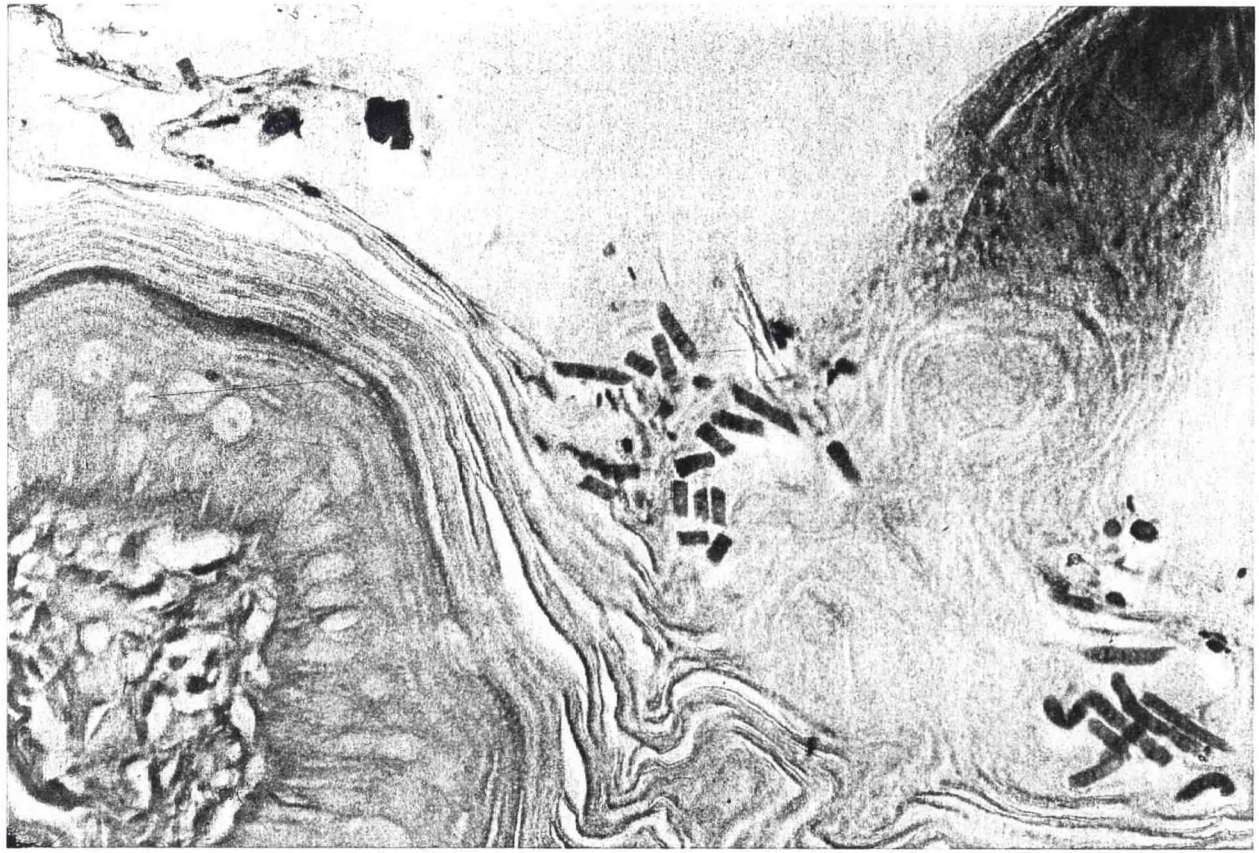

Figure 5.--Unidentified algae and bacteria on the surface of a fibropapilloma of a green turtle (*Chelonia mydas*), Kaneohe Bay, Island of Oahu, Hawaii, 1995. GMS X 40.

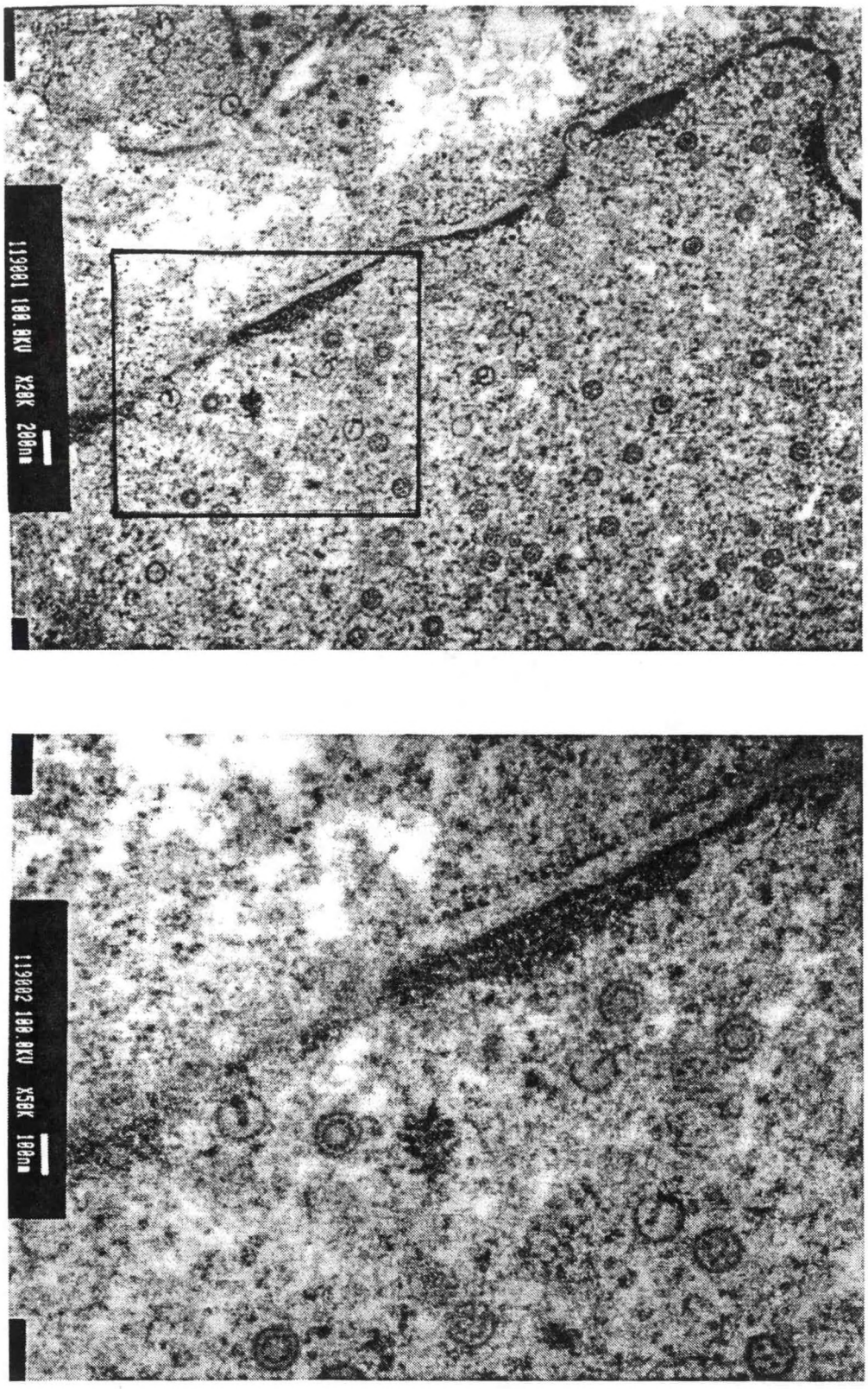


Figure 6.--Herpes virus-like virus identified in the nucleus of a keratinocyte at different stages of replication. Notice the virion budding through the nuclear membrane. X 20 000.