Morphologic and cytochemical characteristics of blood cells from Hawaiian green turtles

Thierry M. Work, MS, DVM, MPVM; Rose E. Raskin, DVM, PhD; George H. Balazs, MS; Scott D. Whittaker, BS

Objective—To identify and characterize blood cells from free-ranging Hawaiian green turtles, *Chelonia mydas*.

Sample Population—26 green turtles from Puako on the island of Hawaii and Kaneohe Bay on the island of Oahu.

Procedure—Blood was examined, using light and electron microscopy and cytochemical stains that included benzidine peroxidase, chloroacetate esterase, alpha naphthyl butyrate esterase, acid phosphatase, Sudan black B, periodic acid-Schiff, and toluidine blue.

Results—6 types of WBC were identified: lymphocytes, monocytes, thrombocytes, heterophils, basophils, and eosinophils (small and large). Morphologic characteristics of mononuclear cells and most granulocytes were similar to those of cells from other reptiles except that green turtles have both large and small eosinophils.

Conclusions—Our classification of green turtle blood cells clarifies improper nomenclature reported previously and provides a reference for future hematologic studies in this species. (*Am J Vet Res* 1998; 59:1252–1257)

As from mammals, blood cells from reptiles can be broadly grouped as RBC, granulocytes, and mononuclear cells. However, classification of blood cells in reptiles is inconsistent because variable criteria have been used to categorize cells or because cellular lineages have been uncertain. In a review,[1] Saint Girons reported 9 types of cells, identified on the basis of light microscopy, that included erythrocytes, eosinophils, basophils, azurophils, neutrophils, lymphocytes, monocytes, thrombocytes, and plasma cells. Sypek and Borysenko[2] reported erythrocytes, eosinophils, heterophils, basophils, monocytes, lymphocytes, and thrombocytes, which were identified on the basis of light microscopy and cytochemical and ultrastructural morphologic characteristics.

Descriptions of morphologic characteristics of blood cells in marine chelonians are limited. Furthermore, classifications are not consistent within a species and when similar methods are used. Wood and Ebanks[3] used light microscopy to classify cells from captive green turtles (*Chelonia mydas*) from the Caribbean as erythrocytes, lymphocytes, eosinophils, basophils, neutrophils, and thrombocytes. However, Aguirre et al,[4] also using light microscopy, classified WBC from Hawaiian green turtles as heterophils, neutrophils, lymphocytes, eosinophils, and basophils. These 2 groups of investigators did not use additional methods, such as cytochemistry and electron microscopy, to corroborate their findings. This may, in part, explain the discrepancy in their classification of WBC, which agrees with that of Saint Girons,[1] who identified neutrophils in reptiles, but contradicts that of Sypek and Borysenko,[2] who did not. Neutrophils have not been commonly documented in reptiles. However, heterophils in reptiles[5] and birds[6] function analogously to mammalian neutrophils.

Clarification of the morphologic characteristics and classification of blood cells, especially WBC, from green turtles is important because this species is intensively scrutinized in captivity in clinical settings and in the wild during research projects. The purpose of the study reported here was to classify blood cells from green turtles by use of light and electron microscopy, and cytochemical techniques.

Material and Methods

Blood procurement and processing—Green turtles were captured while in foraging aggregations at Puako on the west coast of the island of Hawaii (n = 10) and from Kaneohe Bay on Oahu (16). Turtles were hand-captured and brought to shore where they were examined for fibropapillomas on the skin, eyes, and glottis.[7,8] Only those turtles that were clinically normal, immature, in good physical condition, and did not have fibropapillomas were included in this study. To assess stage of maturity, straight carapace length of each turtle was measured to the nearest 0.1 cm, with calipers. Straight carapace length of immature turtles was 48.2 ± 5.6 cm (range, 41 to 59 cm). All turtles weighed > 10 kg.

Ten milliliters of blood was obtained from the cervical sinus[9] in 5-ml heparinized tubes, using sterile syringes and needles. Blood smears were immediately made in duplicate and air dried. Heparinized blood was stored at 4 C for 6 to 8 hours before processing. Hematocrit was determined, and WBC[a] and CBC[10] counts were done. Remaining blood was centrifuged and plasma eluted. The cell layer was overlaid with an equal volume of formalinized glutaraldehyde, and stored at 4 C. After 24 hours, the fixed cell layer was removed and stored in fresh formalinized glutaraldehyde at 4 C until processed for electron microscopy, using standard techniques.

Blood smears were stained with a quick Romanowsky-type stain[b] according to manufacturer's instructions for dif-

Received for publication Oct 17, 1997.
Manuscript passed review Feb 25, 1998.

From the US Geological Survey, Biological Resource Division, National Wildlife Health Center, Honolulu Field Station, PO Box 50167, Honolulu, HI 96850 (Work); the Department of Physiological Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610 (Raskin); the National Marine Fisheries Service, Southwest Fisheries Science Center, Honolulu Laboratory, 2570 Dole St, Honolulu, HI 96822 (Balazs); and the Electron Microscopy Core Laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL 32611 (Whittaker).

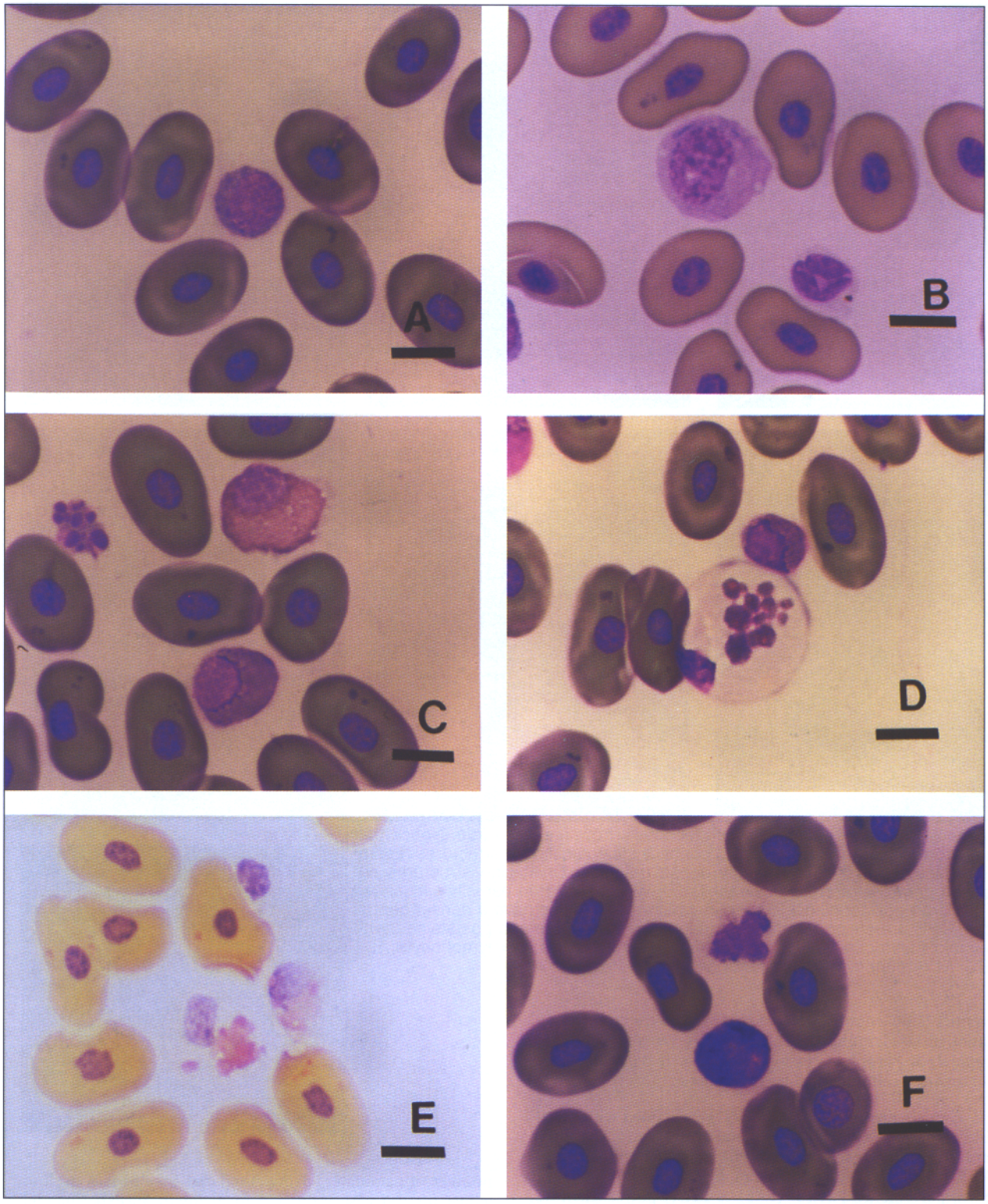

Figure 1—Photomicrographs of blood cells from a green turtle stained with Romanowsky-type stain. Notice a lymphocyte (A), monocyte (B, center) and thrombocyte (B, lower right). Notice red fusiform granules in the cytoplasm of the heterophil (C, top) compared with the more basophilic to orange granules in the small eosinophil (C, bottom). Fragmented nuclear debris are seen on the left. Notice the small eosinophil (D, top) and the large eosinophil (D, bottom) with large granules in abundant cytoplasm. In eosinophils stained with chloroacetate esterase (E), notice that granules of large (left) and small (right) eosinophils stain red; a nonstained thrombocyte is at the top of the photomicrograph. A basophil (F, bottom) and distorted lymphocyte (top) are seen. Bars = 10 μm

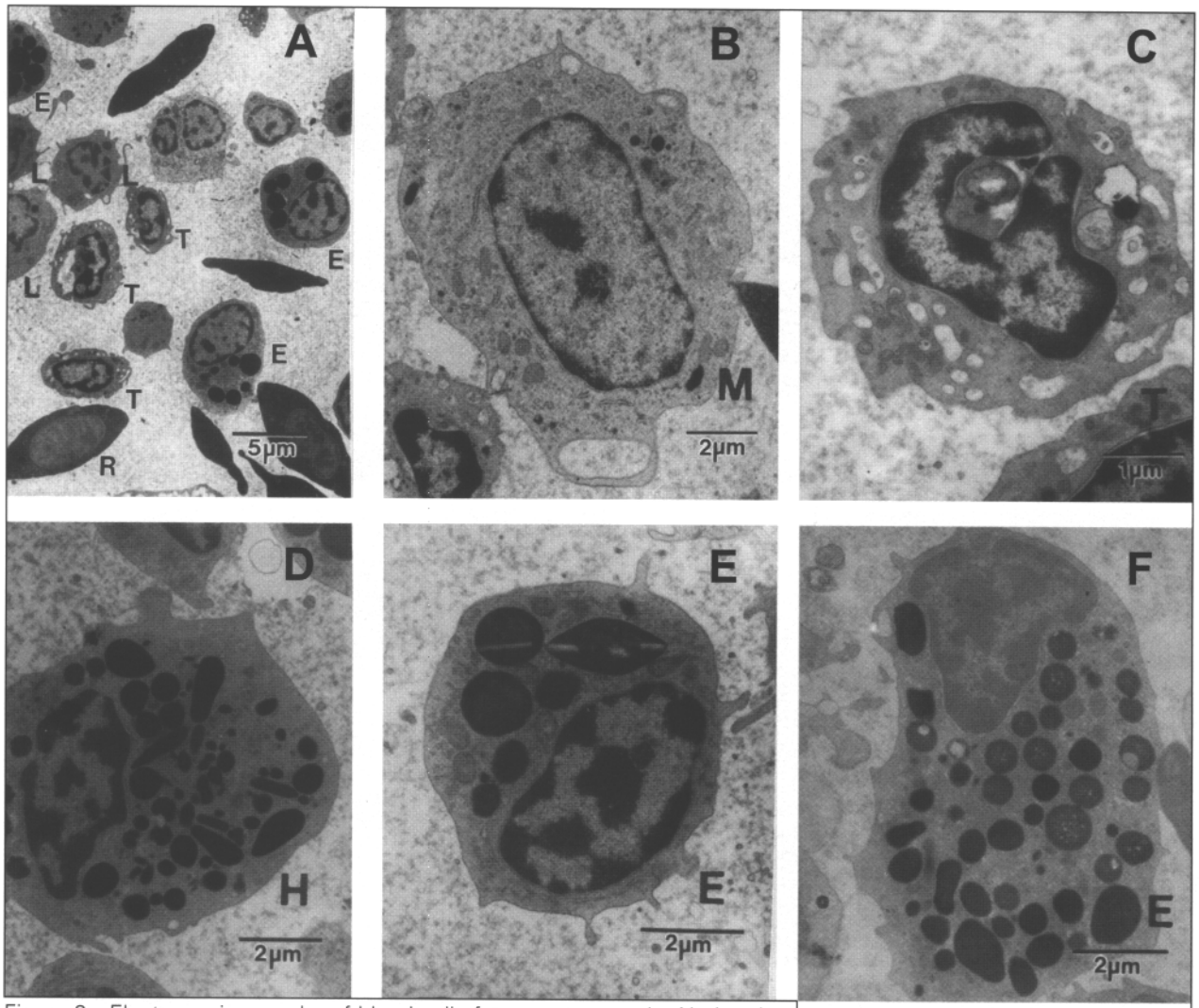

Figure 2—Electron micrographs of blood cells from a green turtle. Notice that the cytoplasm of thrombocytes (C) contains clear canalicular structures resembling vacuoles and small variably dense granules. Crystalline structures are seen in granules of small eosinophils (E), which are often partially vacuolated (F). A large eosinophil (G) is seen in which the granules have a denser central core. Cells are stained with 2% aqueous uranyl acetate and poststained with Reynold's lead citrate. E = eosinophil; T = thrombocyte; L = lymphocyte; R = RBC; M = monocyte; H = heterophil.

ferential WBC count. Two hundred WBC were counted and classified as lymphocytes, monocytes, eosinophils, heterophils, or basophils. Twenty of each cell type were measured with a calibrated ocular micrometer.

Cytochemistry—Blood smears were stained with benzidine **peroxidase (PER)**, **chloroacetate esterase (CAE)**, alpha **naphthyl butyrate esterase (NBE)**, and **acid phosphatase (ACP)** to detect enzymes, **Sudan black B (SBB)** to detect lipids, **periodic acid-Schiff (PAS)** to detect polysaccharides, and **toluidine blue (TB)** to detect sulfated proteoglycans.[11] Positive controls were stained concurrently to ensure adequate staining procedure. For each type of WBC, extent and pattern of staining were recorded.

Data analysis—Means of the absolute count of each cell type, Hct, and WBC count for turtles from Puako versus Kaneohe Bay were compared, using a Student's *t*-test. A sequential Bonferroni adjustment[12] for α of 0.008 for the number of parameters being compared (n = 6) was used to adjust for test-wise error and to maintain an experimental error rate of 0.05. The Mann-Whitney *U* test was used when the assumption of normality or equal variance was not valid.

Results

Erythrocytes were oval with a pale green-orange cytoplasm and a purple-blue oval nucleus. Erythrocyte were 17 to 20 µm long. Numerous erythrocytes had small amorphous intracytoplasmic inclusions near the

Table 1—Differential WBC counts for 26 clinically normal immature green turtles

Cell type	Cell count ($\times 10^3/\mu l$) Mean ± SD	Range
Lymphocyte	10 ± 4.3	3.6–18.6
Monocyte	0.8 ± 0.5	0.1–1.9
Heterophil	1.4 ± 0.8	0.3–3.2
Eosinophil	1.7 ± 0.6	0.7–3.2
Basophil	0.0 ± 0.0	0.0–1.0
Total WBC	13.8 ± 5.3	5.9–23.6

poles of the cell or adjacent to the nucleus. On a wet-mount preparation of blood stained with new methylene blue, these inclusions were pale and did not stain. On electron micrographs, inclusions were pleomorphic and electron-dense without recognizable organelles or nucleus. These inclusions were too large to be viral particles[13] and, because there was no cell wall, distinct cell membrane, or pili, they were not considered to be bacteria. On the basis of these observations, these inclusions were considered degenerating organelles.

Lymphocytes ranged from 6 to 14 µm in diameter, and were small and round with a well-defined round purple-blue nucleus that contained prominently clumped chromatin (**Fig 1**). The nucleus was surrounded by a rim of granular basophilic cytoplasm that was often blebbed, and the nucleocytoplasmic ratio was typically about 2:1. Lymphocytes did not take up any cytochemical stain. On electron micrographs, the nucleus was round, often indented, and had large amounts of heterochromatin; the scant cytoplasm contained few mitochondria, endoplasmic reticulum, and small electron-dense granules (**Fig 2**).

Monocytes were larger than lymphocytes, and were round or amoeboid and from 11 to 26 µm in diameter (Fig 1). The nucleus was purple-blue, and round, oval, or indented, and had a fine chromatin pattern. The cytoplasm was mildly to moderately basophilic, had well-defined or irregular borders, and contained variably sized intracytoplasmic vacuoles. Azurophilic granules were not seen. Monocytes were stained with ACP and PAS but only faintly or not all with CAE and NBE. By electron microscopy, the nuclei of monocytes had scant heterochromatin and nucleoli were sometimes found. The abundant cytoplasm contained a large golgi zone, mitochondria, endoplasmic reticulum, and a few small dense granules (Fig 2).

Thrombocytes (Fig 1) were oval and smaller than erythrocytes (9 to 12 µm long). The nucleus was oval to angular and homogeneously dense purple-blue, and was surrounded by a thin rim of clear cytoplasm. Thrombocytes only stained with NBE and PAS when they appeared as multiple punctate densities. By electron microscopy (Fig 2), these cells were oval to fusiform with irregular oval to lobulated purple nuclei that contained abundant heterochromatin. Cell borders had slender finger-like projections, and the cytoplasm often contained clear canalicular structures and small uniform membrane-lined granules

Heterophils were 10 to 18 µm in diameter and were characterized by dense, round to oval, purple-blue, often eccentric nuclei. The cytoplasm was filled with numerous fusiform dull-red granules (Fig 1). Heterophils were stained with NBE and the cytoplasm was stained with PAS. On electron micrographs, nuclei had moderate amounts of heterochromatin. The cytoplasm contained numerous electron-dense, elongate to round granules and smaller numbers of pleomorphic, variably dense granules. Endoplasmic reticulum and small numbers of mitochondria were noticed (Fig 2).

Eosinophils were of 2 types (Fig 1): small, which were 12 to 16 µm long and had well-defined, round, dense purple-blue nuclei; and large, which were 14 to 22 µm and had amorphous, purple-blue, eccentric nuclei. Small eosinophils had a blue cytoplasm that contained numerous well-defined round granules that were dull orange or clear. The latter type of granule gave the cell the appearance of a vacuolated lymphocyte. Variably sized, well-defined metachromatic granules were uncommonly seen. Large eosinophils had abundant clear to lightly granular cytoplasm that contained sparse large round granules. These granules had a bright orange core and were surrounded by a blue-orange slightly refractile halo. Granules in both types of eosinophils stained strongly with CAE and marginally with NBE and PAS.

Morphologic characteristics of eosinophils were similar by light and electron microscopy. The nucleus was round to oval and contained variable amounts of heterochromatin. Small eosinophils had well-defined round dense homogeneous granules, which often contained elongate crystalline structures that deformed the granule (Fig 2). A few eosinophils also had numerous variably dense granules, which often contained small multiple to large single clear vacuoles. Large eosinophils had numerous coalescing, clear, poorly defined small vacuoles in abundant cytoplasm. Few large, variably dense, round to pleomorphic, well-defined granules were found on one end of the cell. A few of these granules contained an electron-dense core that resembled crystalloid granules found in other species but lacked lamellar periodicity. Mitochondria, endoplasmic reticulum, and golgi zones were most notable in small eosinophils.

Basophils (Fig 1) consisted of a purple-blue dense nucleus and a well-defined cytoplasm that contained numerous small basophilic granules that obscured the nucleus. Basophil granules stained with TB. Measurements were not made and ultrastructural characteristics were not determined because this cell was rare.

Hematocrit and differential counts—Mean ± SD of Hct was 29 ± 4% (range, 17 to 35%). White blood cell counts were not significantly different between turtles captured in Puako versus Kaneohe Bay; therefore, values for these 2 groups were pooled. Data are given as mean ± SD and range (**Table 1**).

Discussion

Morphologic characteristics of RBC from green turtles in this study were similar to those reported for green turtles[3] and other chelonians.[2,14] Degenerating organelles in the cytoplasm of RBC from green turtles were similar to those found in RBC from desert tortoises.[14]

We classified 6 types of WBC in blood from green

turtles, which is similar to the classification of WBC from reptiles proposed by Sypek and Borysenko.[2] As in many terrestrial and marine reptiles,[2,3,15] lymphocytes were the most numerous circulating WBC from green turtles. Morphologic characteristics of lymphocytes from green turtles were similar to those from other marine chelonians except that cytoplasmic vacuoles were not seen; this is in contrast to findings of Woods and Ebanks.[2] Cannon[15] found that lymphocytes from Kemp's Ridley turtles stained with nonspecific esterase; however, lymphocytes from green turtles in this study did not stain with all the cytochemical stains that we used, which is similar to the staining characteristics for lymphocytes from desert tortoises.[14] Ultrastructural morphologic characteristics of lymphocytes from green turtles was similar to those of lymphocytes from other reptiles.[2,16]

By light microscopy, thrombocytes from green turtles were similar to those from other reptiles.[2] Cannon[15] did not describe thrombocytes in Kemp's Ridley turtles, whereas Wood and Ebanks[3] found that it was difficult to differentiate thrombocytes from basophils in green turtles. Although thrombocytes from other reptilian species can be difficult to distinguish from lymphocytes,[14,16] we found that, on freshly prepared smears, thrombocytes usually retained their morphologic characteristics. However, if smears were prepared from blood that had been chilled, cellular shrinkage made differentiation more difficult. Thrombocytes were differentiated from lymphocytes cytochemically by staining with NBE and PAS. Unlike terrestrial reptiles,[2,16] green turtles had thrombocytes that stained with PAS but not with ACP. Ultrastructurally, thrombocytes from green turtles had open canalicular systems, which is a characteristic of this cell type in many reptiles.[2] Platelets in mammals, which have an analogous function to reptilian thrombocytes, also have open canalicular systems.[17]

On light and electron microscopic examination, monocytes from green turtles were similar to those from other reptiles.[2,16] Monocytes have not been documented in blood from Kemp's Ridley turtles[15] or green turtles.[3,4] Handling of blood samples may have explained this discrepancy in green turtles. Monocytes were more difficult to differentiate from lymphocytes when smears were made from blood that had been chilled for 8 hours, partly because of cellular shrinkage. Monocytes in green turtles were cytochemically differentiated from other cells because they stained with ACP and PAS. Unlike desert tortoises,[14] green turtles had monocytes that did not stain with SBB and PER.

On examination, using light microscopy, heterophils from green turtles had cytoplasm that was filled with fusiform red granules, which, on ultrastructural examination, appeared as elongate electron-dense granules similar to those seen in heterophils from other reptiles.[2,16] Heterophils have not been documented in Kemp's Ridley and green turtles.[3,4,15] However, on the basis of light microscopic descriptions, large eosinophils from Kemp's Ridley turtles described by Cannon[15] may have been heterophils. Cytochemical staining can be variable in heterophils from reptiles.[2] Unlike heterophils from terrestrial reptiles, which stain with ACP and ALP,[2,14] heterophils from green turtles only stained with NBE and PAS.

Heterophils in reptiles[5] and birds[6] have a role that is analogous to that of the mammalian neutrophil. Classifying reptilian WBC as neutrophils[1,3] is incorrect if these cells contain intracytoplasmic fusiform red granules. We did not identify circulating neutrophils in blood from green turtles. Criteria were insufficient for us to judge the merit of what others[4] have described as neutrophils in green turtles. On the basis of photomicrographs, we suspect that neutrophils described by Wood and Ebanks[3] were actually large eosinophils that had degranulated. Neutrophils, which are characterized by a segmented nucleus and do not have cytoplasmic granules, are rare in reptiles but have been described in tuataras (*Sphenodon punctatus*).[18]

Eosinophils from green turtles had intracytoplasmic round dull-orange, often vacuolated granules, as do eosinophils from other reptilian species.[2,15] Vacuolated lymphocytes described by Woods and Ebanks[3] in blood from green turtles may have been small eosinophils with vacuolated granules. Vacuolated eosinophils are common in certain dog breeds such as Greyhounds.[19] Although small eosinophils with pale granules resembled vacuolated lymphocytes, they were considered eosinophils on the basis of the presence of crystalline material within granules on electron microscopic examination. Crystalline material in granules is rarely found in reptilian eosinophils[2] but is a distinguishing characteristic in some mammalian eosinophils.[19] Finally, eosinophils are often seen in response to parasitic infections.[19,20] On histologic examination of tissues from green turtles from Hawaii that were infected with vascular flukes, large eosinophils and mononuclear cells were the predominant inflammatory cell types observed.[c] This would presumably give further support that these large cells are eosinophils.

Large and small eosinophils are uncommonly documented in reptiles.[15] We suspect that large eosinophils in green turtles represent activated cells that contain degranulated or coalescent granular material in response to a parasitic infection or other inflammatory stimulus. We do not have data indicating whether large eosinophils are more or less mature than small eosinophils. Microscopic examination of granulopoietic sites could resolve this dilemma. Unlike those from other reptiles,[2] eosinophils from green turtles in this study were only stained with CAE.

Contrary to the findings of others,[3,4] we found that basophils were rare in green turtles. Possible reasons for this discrepancy include seasonal, geographic, or age variation. Cannon[15] did not identify basophils in Kemp's Ridley turtle.

Hematocrits and WBC count from green turtles in our study were lower than those determined by Woods and Ebanks[3] but within range of those determined by Aguirre et al.[4] This may be attributable in part to differ-

ences in methods used. Wood and Ebanks[3] used a 1% Wrights stain in saline (0.85% NaCl) solution as a stain, 5% formalin as a diluent, and counted cells manually, whereas we used the solution contained within commercially available cuvettes as our diluent. Aguirre et al[4] used a Coulter counter to quantify WBC from green turtles. Even if consistent methods are used, WBC counts in reptiles may be highly variable; thus, they may be most useful in comparative studies or when monitoring progress of a reptile in a clinical case.[10]

[a]Unopette No. 5877, Becton-Dickinson, Rutherford, NJ.
[b]Leukostat, Fisher, Pittsburgh, Penn.
[c]Work TM, the US Geological Survey, Biological Resource Division, National Wildlife Health Center, Honolulu Field Station, Honolulu, HI: Unpublished data, 1997.

References

1. Saint Girons MC. *Morphology of the circulating blood cells*. In: Gans C, ed. London: Academic Press Inc, 1970;73–90.

2. Sypek J, Borysenko M. Reptiles. In: AF Rowley, NA Ratcliffe, eds. *Vertebrate blood cells*. Cambridge, England: Cambridge University Press, 1988;211–256.

3. Wood FE, Ebanks GK. Blood cytology and hematology of the green sea turtle, *Chelonia mydas*. *Herpetologica* 1984;40:331–336.

4. Aguirre AA, Balazs GH, Spraker TR, et al. Adrenal and hematological responses to stress in juvenile green turtles (*Chelonia mydas*) with and without fibropapillomas. *Physiol Zool* 1995;68: 831–854.

5. Montali RJ. Comparative pathology of inflammation in the higher vertebrates (reptiles, birds and mammals). *J Comp Pathol* 1988;99:1–26.

6. Brooks RL Jr, Bounous DI, Andreasen CB. Functional comparison of avian heterophils with human and canine neutrophils. *Comp Haematol Int* 1996;6:153–159.

7. Balazs GH, Forsyth RG, Kam AKH. Preliminary assessment of habitat utilization by Hawaiian green turtles in their resident foraging pastures. In: *NOAA Technical Memorandum NMFS, NOAA-TM-NMFS-SWFC-71*. Honolulu: US Department of Commerce, 1987;107.

8. Balazs GH, Pooley SG. Research plan for marine turtle fibropapilloma. In: *NOAA Technical Memorandum NMFS*, NOAA-TM-NMFS-SWFC-156. Honolulu: US Department of Commerce 1991;113.

9. Owen DW, Ruiz GJ. New methods of obtaining blood and cerebrospinal fluid from marine turtles. *Herpetologica* 1980;36:17–20.

10. Campbell TW. Clinical pathology. In: DR Mader, ed. *Reptile medicine and surgery*. Philadelphia: WB Saunders Co, 1996;248–257.

11. Jain NC. Cytochemistry of normal and leukemic leukocytes. In: *Schalm's veterinary hematology*. 4th ed. Philadelphia: Lea & Febiger, 1986;909–939.

12. Rice WR. Analyzing tables of statistical tests. *Evolution* 1989;43:223–225.

13. Cheville NF. *Cell pathology*. Ames, Iowa: Iowa State University Press, 1983;451–502.

14. Alleman AR, Jacobson ER, Raskin RE. Morphologic and cytochemical characteristics of blood cells from the desert tortoise (*Gopherus agassizii*). *Am J Vet Res* 1992;53;1645–1651.

15. Cannon MS. The morphology and cytochemistry of the blood leukocytes of Kemps Ridley sea turtle (*Lepidochelys kempi*). *Can J Zool* 1992;70:1336–1340.

16. Bounous DI, Dotson TK, Brooks RL Jr, et al. Cytochemical staining and ultrastructural characteristics of peripheral blood leukocytes from the yellow rat snake (*Elaphe obsoleta quadrivitatta*). *Comp Haematol Int* 1996;6:86–91.

17. Jain NC. The platelets: structural, biochemical and functional aspects. In: *Schalm's veterinary hematology*. 4th ed. Philadelphia: Lea and Febiger, 1986;446–465.

18. Desser SS. Morphological, cytochemical, and biochemical observations on the blood of the tuatara, *Sphenodon punctatus*. *New Zealand J Zool* 1978;5:503–508.

19. Jain NC. The eosinophils. In: *Schalm's veterinary hematology*. 4th ed. Philadelphia: Lea and Febiger, 1986;731–755.

20. Glazebrook JS, Campbell RSF, Blair D. Pathological changes associated with cardiovascular trematodes (Digenea: Spirorchidae) in a green sea turtle, *Chelonia mydas* (L.). *J Comp Pathol* 1981;91: 361–368.