Inclusion Body Disease, A Worldwide Infectious Disease of Boid Snakes: A Review

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Abstract

A disease called *inclusion body disease* (IBD) is seen worldwide in snakes that are members of the families Boidae and Pythonidae. Snakes affected by this disease often have neurological signs. A diagnosis is based on light microscopic examination of tissues for the presence of intracytoplasmic inclusions that consist of a unique protein that has been termed *inclusion body disease protein* (IBDP). In some cases, inclusions are found exclusively in the central nervous system, whereas in others they may be diffusely disseminated in multiple tissues. In a few cases, the IBD inclusions have overlapped in appearance with other types of nonviral intracytoplasmic inclusions. The specific etiologic agent of IBD remains unknown. Because the underlying cause of IBD is unknown, recent research has focused on understanding the formation and nature of IBDP. A monoclonal antibody has been developed against IBDP and is currently being used in immunodiagnostic assays to develop a reliable diagnostic test for IBD. This monoclonal antibody is going through rigorous validation testing and will provide more specificity and sensitivity than currently available routine histological diagnostics. Because large numbers of boas and pythons are being bred and sold for the pet trade, better diagnostic tests are needed to help develop IBD-free breeding colonies of these snakes. Further, because IBD is one of the few worldwide diseases of captive snakes, there is concern in many countries (e.g., Australia, where it has been identified in captive pythons) that it will become established in native wild populations. Thus, there is conservation value in developing better diagnostic tools for screening snakes intended for release as part of reintroduction programs.

Key words: **Boa; IBD; IBDP; monoclonal antibody; protein sequencing; python**

Snakes make up approximately 19% of all reptiles maintained as pets in the U.S.[1] Of these, various boid snakes (members of the families Boidae and Pythonidae) are bred in large numbers for the pet trade. Although accurate numbers are not available, it is believed that several million of these snakes are classified as pets or maintained in breeding operations within the United States. Of illnesses affecting boid snakes, inclusion body disease (IBD) has surfaced as the most important world-

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wide disease, a condition characterized by the formation of intracytoplasmic inclusions.[2] In Australia, where IBD has been identified in captive pythons,[3] and in other countries where boid snakes are being bred for release to the wild, there is concern that this disease will become established in native wild populations. Boid snakes with IBD may have a subclinical infection. It is not known what percentage of infected snakes will develop clinical signs of disease in relation to those that will appear unaffected. It is possible that latent infections can persist for long periods of time. Currently, a presumptive diagnosis of IBD is based on the light microscopic identification of intracytoplasmic inclusions in one or more tissues. However, some snakes have very few inclusions in tissue sections because they are easy to overlook, especially if limited to the central nervous system (CNS). Although several viruses, including retroviruses,[4] have been identified and isolated from snakes with IBD, the causative agent remains unknown. Because the inclusions consist of a unique protein (inclusion body disease protein, IBDP),[5] understanding the cause of IBD will be based on determining the composition and factors affecting the formation of this protein. To better understand the nature of IBDP, this protein needs to be entirely or partially sequenced. Eventually the sequencing of this protein will allow the creation of peptides that can be used in the development of better immunodiagnostic tests to screen individuals and colonies of snakes for IBDP.

History, Hosts, and Geographic Range

In the 1970s, IBD was first identified in the United States, where it affected multiple species of boid snakes in private and zoological collections.[2] When first recognized, Burmese pythons (*Python bivittatus*) were the most common boid snake diagnosed with IBD. In 1998, IBD was reported in captive native carpet (*Morelia spilota variegata*) and diamond pythons (*M. spilota spilota*) in Australia,[3] in captive boa constrictors in the Canary Islands, Spain,[6] and subsequently in Belgium.[7] Beginning in the early 1990s, more cases of IBD were diagnosed in boa constrictors than pythons, but the cause of this epidemiologic shift is unknown. Additional species diagnosed with IBD include the green anaconda (*Eunectes murinus*), yellow anaconda (*Eunectes notaeus*), rainbow boa (*Epicrates cenchris*), Haitian boa (*Epicrates striatus*), Madagascan boa (*Acranthophis madagascariensis*), Indian python (*P. molurus molurus*), reticulated python (*P. reticulatus*), and ball python (*P. regius*). In addition, a disease resembling IBD was diagnosed in an eastern king snake (*Lampropeltis getula*) that was housed with boa constrictors,[4] and in a zoological collection of palm vipers (*Bothriechis marchi*).[8] However, the correlation of the inclusions in the king snake and viper cases to IBD has not been confirmed with molecular techniques or immunological reagents.

Clinical Signs

From the late 1970s and extending into the mid-1980s, Burmese pythons were the most common boid snake seen with IBD. Clinical signs of the disease in Burmese pythons primarily involved CNS abnormalities (e.g., torticollis, disequilibrium, opisthotonos, inability to right itself when placed in dorsal recumbency, flaccid paralysis).[2] Beginning in the early 1990s, more cases were diagnosed in boa constrictors in relation to Burmese and other pythons. Boa constrictors affected by IBD also regurgitated food items within several days of feeding, in addition to the CNS disease signs described for pythons (Figs 1 and 2). Although some snakes die within several weeks of first manifesting illness, others may survive for months. Other clinical signs observed in affected snakes were stomatitis, regurgitation, pneumonia, lymphoproliferative disorders, and round cell tumors. Regurgitation was not a disease sign identified in Burmese pythons.

Hematological and selected biochemical analyte values of acutely affected boa constrictors diagnosed with IBD included leukocytosis, relative lymphocyto-

Figure 1. Boa constrictor. Boidae. IBD. This abnormal posture is a sign of CNS disease.

Figure 2. Boa constrictor. IBD. When placed in dorsal recumbency, this snake was unable to right itself. Courtesy of CRC Press.

sis, lower total protein and globulin values, and significantly higher aspartate transaminase values compared with those of chronically affected snakes.[2]

Postmortem Diagnosis

A postmortem diagnosis of IBD is based on the light microscopic identification of variably sized eosinophilic to amphophilic intracytoplasmic inclusions in hematoxylin and eosin (H&E)–stained tissue sections. The tinctorial characteristics of the inclusions may vary with the type of hematoxylin used and differences in staining methods.[9] In pythons, inclusions are mostly found within neurons in the CNS (Figs 3 and 4). In boa constrictors, inclusions are also commonly observed in neurons and glial cells in

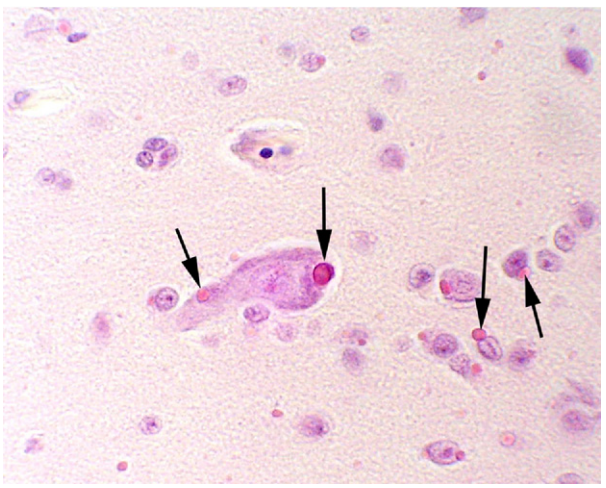

Figure 3. Boa constrictor. IBD. Photomicrograph of eosinophilic intracytoplasmic inclusions (arrows) in neurons and glial cells in the brain. H&E stain.

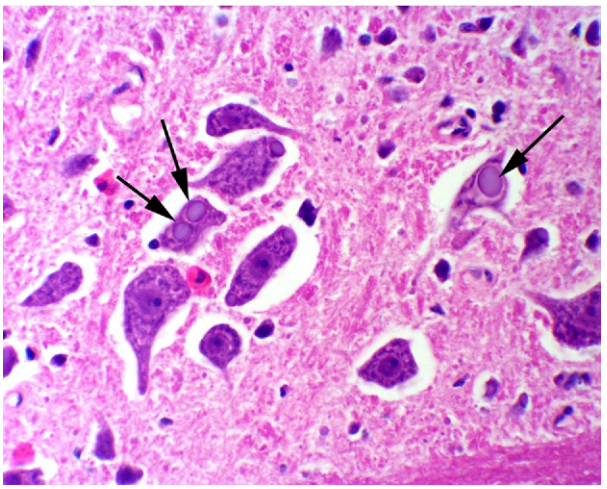

Figure 4. Boa constrictor. IBD. Photomicrograph of amphophilic intracytoplasmic inclusions in neurons of the brain. H&E stain. Courtesy of Nikos Gurfield and CRC Press.

the CNS, with or without an associated inflammation. If identified, encephalitis is generally more severe in pythons compared with that in boa constrictors. In boa constrictors, inclusions also are commonly seen in: 1) mucosal epithelial cells adjacent to and overlying esophageal tonsils, 2) lymphoid cells in esophageal tonsils (Fig 5), 3) epithelial cells lining the gastrointestinal tract, 4) epithelial cells lining the respiratory tract, 5) hepatocytes (Fig 6), 6) pancreatic acinar cells (Fig 7), and 7) renal tubular epithelial cells.

Electron Microscopic Findings

Using transmission electron microscopy, intracytoplasmic inclusions identified within CNS visceral epithelial cells and nerve cells begin as polyribosome-

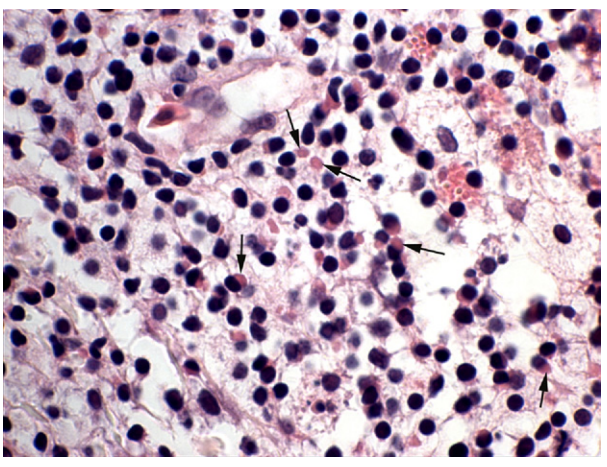

Figure 5. Boa constrictor. IBD. Photomicrograph of an esophageal tonsil from a necropsied snake showing numerous eosinophilic intracytoplasmic inclusions (arrows) within submucosal lymphoid cells. H&E stain. Courtesy of CRC Press.

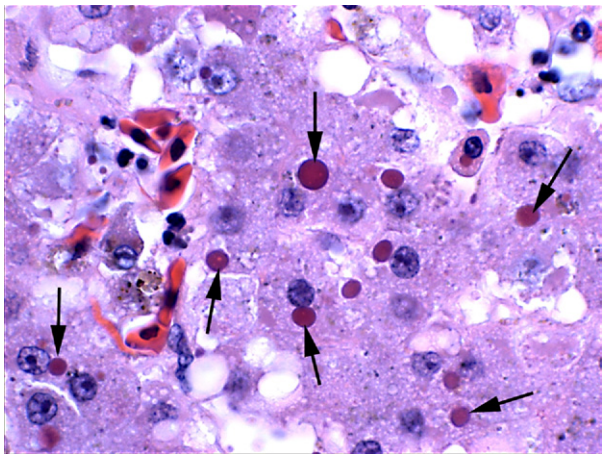

Figure 6. Boa constrictor. IBD. Photomicrograph of the liver showing hepatocytes containing eosinophilic intracytoplasmic inclusions (arrows). H&E stain. Courtesy of CRC Press.

derived clusters of small round subunits (Fig 8).[10] Inclusions that enlarge as additional subunits are deposited on the periphery of individual inclusions (Fig 9). In some sections the inclusions have concentric profiles, with subunits observed on the surface. A unique protein (IBDP) was identified as a 68-kd band on a protein electrophoretogram of IBD-infected tissues.[5] Although in some cases the subunits have an ultrastructural appearance resembling viral particles, the current findings indicate that the inclusions are nonviral and mainly consist of IBDP. Beyond this protein, the chemical composition of the inclusions remains unknown.

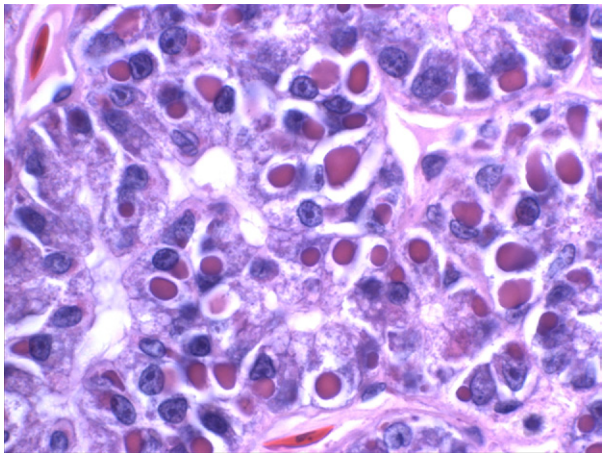

Figure 7. Boa constrictor. IBD. Photomicrograph of the pancreas showing acinar cells containing eosinophilic intracytoplasmic inclusions. H&E stain. Courtesy of CRC Press.

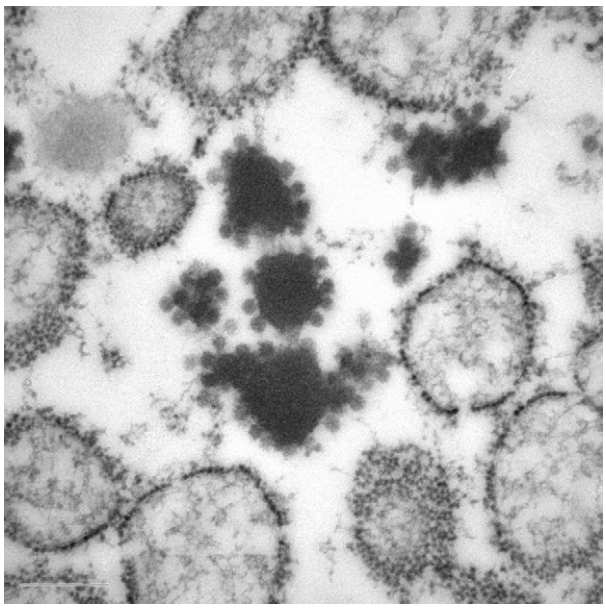

Figure 8. Boa constrictor. Transmission electron photomicrograph of an enterocyte in the small intestine of a snake with inclusion body disease. During the initial stage of inclusion formation, protein subunits from polyribosomes start accumulating in the adjacent cytoplasm. Uranyl acetate and lead citrate stain. Courtesy of CRC Press.

Antemortem Diagnosis

An antemortem diagnosis is made by demonstrating eosinophilic to amphophilic intracytoplasmic inclu-

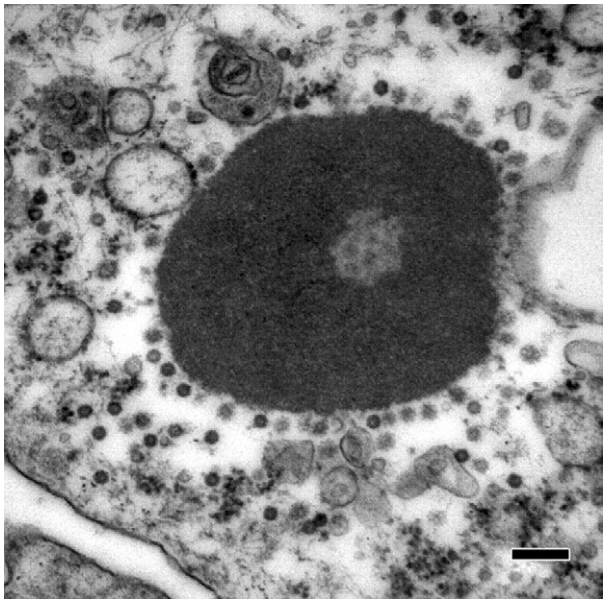

Figure 9. Boa constrictor. Transmission electron photomicrograph of an inclusion in an enterocyte. Deposited protein subunits have a virus-like appearance. Uranyl acetate and lead citrate stain. Courtesy of CRC Press.

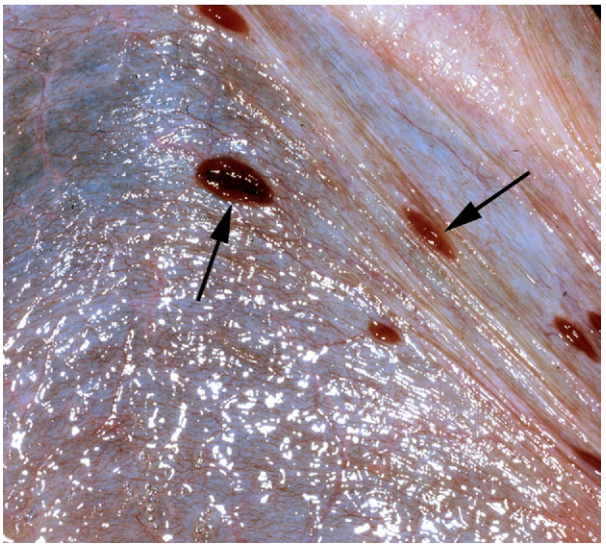

Figure 10. Reticulated python. Pythonidae. Esophageal tonsils (arrows) are raised ovoid structures with a central cleft and covered by a mucous epithelium. Courtesy of CRC Press.

sions in histologically processed and H&E-stained biopsy specimens. Boid snakes have well-developed esophageal tonsils[11] (Fig 10), and in snakes with IBD the tonsils may contain lymphoid cells or mucous epithelial cells with intracytoplasmic inclusions (Fig 5). Using a flexible endoscope with a biopsy device, esophageal tonsils are easily biopsied, fixed, and routinely processed for light microscopy. Liver and kidney biopsy specimens can also be obtained for histological evaluation. For a more rapid diagnosis, cytological impression smears of the liver and renal biopsy samples can be stained with H&E (Table 1; Fig 11) and/or Wright-Giemsa (Fig 12).[12,13] In the authors' experience, inclusions are easier to identify in H&E-stained preparations. Inclusions may be seen in erythrocytes (Fig 13), lymphocytes (Figs 13 and 14), and heterophils (Fig 15) in peripheral blood films of snakes with IBD.

Table 1. Hematoxylin and eosin–staining recommendations for impression smears and blood films

1. Fix 1 minute in 10% neutral buffered formalin
2. Stain 3 minutes in Harris hematoxylin
3. One dip into acid alcohol
4. Briefly wash in running water
5. Dip into 0.5% ammonia to blue the nuclei
6. Wash in running water
7. Counterstain in eosin for 40 seconds
8. Dehydrate in a series of alcohols: 95%-100%-100%
9. Place in xylene and mount as with a paraffin-embedded section

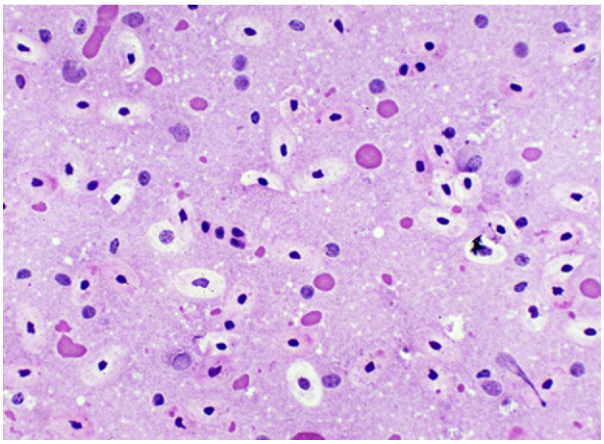

Figure 11. Boa constrictor. IBD. Photomicrograph of a cytological impression of liver. Numerous eosinophilic intracytoplasmic inclusions can be seen. H&E stain. Courtesy of CRC Press.

Cost can be a limiting factor in using the diagnostic test above. Tissue biopsies from living snakes are often processed and evaluated for less than US $150, and full necropsy examinations on dead snakes may cost US $300-$500 per case. Blood smears from suspect cases can be examined for presence of inclusions in lymphocytes and heterophils, but it is not known how many snakes that are diagnosed with IBD also have inclusions in circulating white blood cells. Nonetheless, submission of a blood smear to deter-

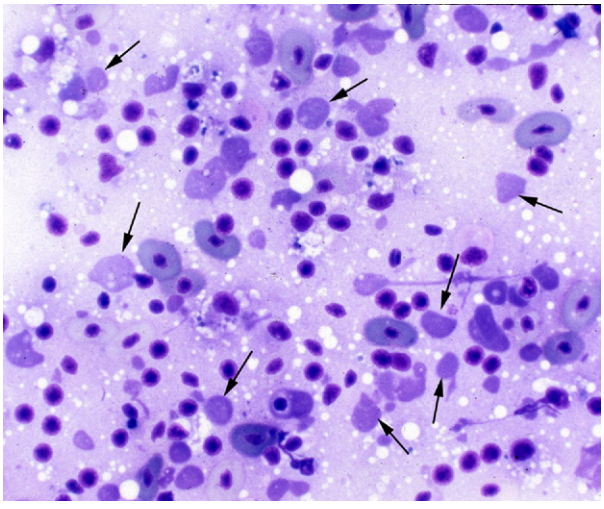

Figure 12. Boa constrictor. IBD. Photomicrograph of cytological impression of liver. Numerous basophilic intracytoplasmic inclusions (arrows) can be seen. Wright-Giemsa stain.

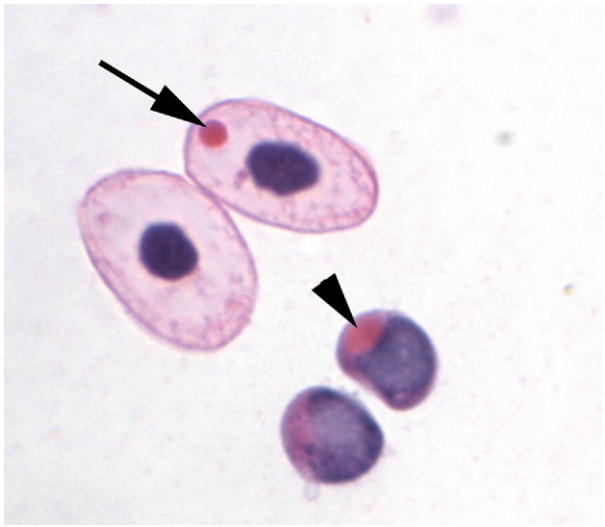

Figure 13. Boa constrictor. IBD. Peripheral blood film with an erythrocyte (arrow) and lymphocyte (arrowhead) containing eosinophilic-staining inclusions. H&E stain.

mine if inclusions are present is relatively inexpensive (approximately US $50).

Cause and Transmission

Retroviruses have been isolated from boas with IBD, and several of these viruses have been partially characterized.[4] Using transmission electron microscopy, viral morphogenesis in cell culture was described.[4] Evaluation of viper heart 2 (VH2) cells infected with

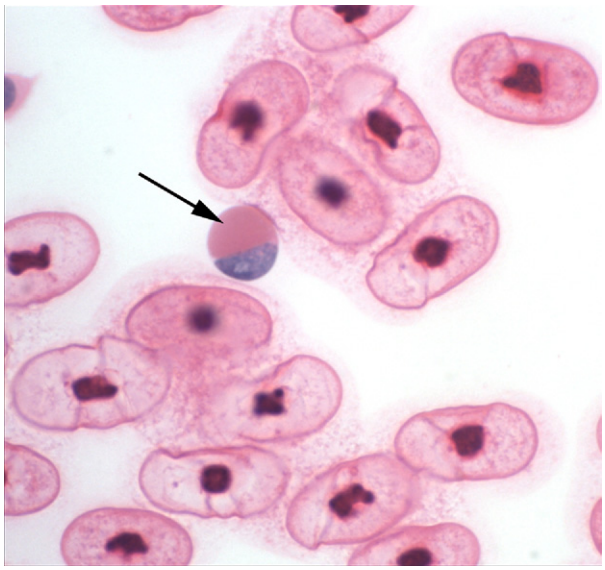

Figure 14. Boa constrictor. IBD. Peripheral blood film with a lymphocyte containing an eosinophilic-staining inclusion (arrow). H&E stain.

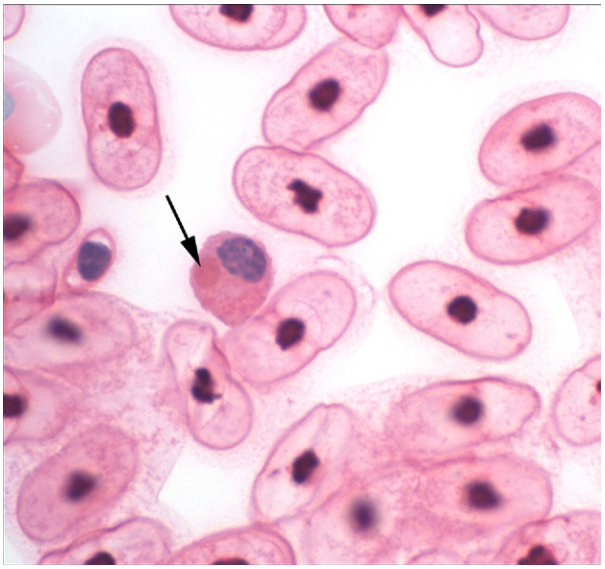

Figure 15. Boa constrictor. IBD. Peripheral blood film with a heterophil containing an eosinophilic-staining inclusion (arrow). H&E stain.

tissues from IBD-positive snakes revealed intracytoplasmic and extracellular virions (Fig 16). Based on size (80-110 nm) and morphology, the virus resembled C-type retroviruses. Reverse-transcriptase activity was measured in infected cell cultures, and high activity levels were further evidence that the isolated virus was a retrovirus. Retroviruses also have been observed in transmission electron microscopy of tissue sections from IBD-infected snakes.[4,5,7] However, the VH-2 cells that were infected with the virus did not form inclusions. Huder and coworkers isolated and sequenced endogenous retroviruses from Bur-

Figure 16. Boa constrictor. Transmission electron photomicrograph of primary kidney cells from a snake with IBD. Extracellular retroviral particles are seen on the surface of kidney cells. Uranyl acetate and lead citrate. Courtesy of CRC Press.

mese and blood pythons (*P. curtus*).[14] The Burmese pythons from which the endogenous retroviruses were sequenced belonged to a collection of snakes in which clinical signs of IBD had been identified, whereas no background information was provided for the blood pythons. Other species of boid snakes tested, whether they had IBD or not, were virus negative. Therefore, it was concluded that there was no causal link between this virus and IBD.[14]

Although retroviruses have been observed in inclusion-bearing tissues, many hours of investigation, using the electron microscope, are needed to locate mature retroviral particles. For a number of IBD cases, neither viral particles nor reverse-transcriptase activity have been demonstrated in tissues of affected snakes. Transmission studies have been performed in Burmese pythons and boa constrictors by inoculating young Burmese pythons with the supernatant of primary cultured kidney cells taken from an infected boa constrictor, resulting in the development of clinical signs and microscopic lesions associated with IBD.[2] In a second study, boa constrictors that were administered filtered liver homogenate obtained from an IBD-infected boa eventually developed intracytoplasmic inclusions in hepatocytes.[5] Because purified virus was not used in these studies, it is impossible to implicate a retrovirus as the underlying etiology of inclusion formation in the inoculated snakes. IBD may represent a protein-storage disease induced by viral infection, or the protein itself may be behaving in a manner similar to that of a prion-like disease. The protein and the isolated viruses must be sequenced to gain a better understanding of a possible causal relationship.

The route of transmission of IBD between snakes has not been determined, although it is believed that direct contact is involved. Because the snake mite (*Ophionyssus natricis*) (Fig 17) is present in many snake colonies experiencing an IBD outbreak, mites may be associated in the transmission of the infectious agent. Thus, preventing mites from entering a collection and eliminating established infestations are essential components of a preventive medicine program. It is also possible the causative agent is passed through vertical transmission from mother to young in both egg-laying and live-bearing snakes.

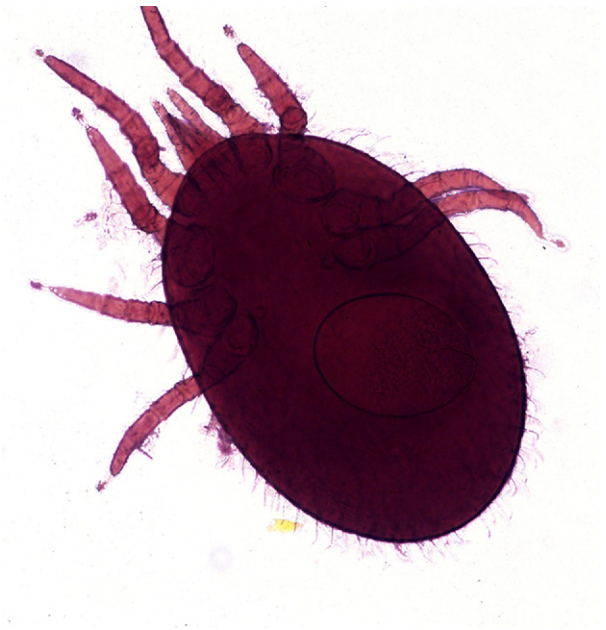

Figure 17. Snake mite, *Ophionyssus natricis*. Photomicrograph of a mite removed from a snake. A single egg can be seen within the mite. Courtesy of CRC Press.

IBD Risk Reduction

There is no simple protocol to follow when trying to prevent IBD from entering a snake collection or managing a breeding operation where the disease has been diagnosed. However, several approaches can reduce the risk of introducing IBD into a snake collection. First and foremost, a solid preventative medicine program should be established. Although no such program is 100% effective, the main objective is reducing the risk of IBD entering a collection. Having a veterinarian knowledgeable about reptiles or specializing in reptile medicine is also a very important component to a preventative medicine program.

It is very helpful to have knowledge about the history of the previous owner or breeder's collection from which the purchased snake is derived. Procuring information regarding the history of a breeding operation, including annual mortality and causes of mortality in a collection, is often difficult or impossible to uncover. Because of the ease at which snakes are sold over the internet and purchased at reptile expositions, new snake "breeders" establish themselves, seemingly, on a daily basis. Based on the authors' experience, the vast majority of snakes that die in a collection never have a thorough necropsy examination. Every collection inevitably has animals that become ill and die, with no collection being pathogen-free. Therefore, buyers of new animals should reduce the risk of introducing an infectious agent by purchasing animals from well-established, recognized breeders that have preventive medicine programs in place rather than purchasing animals because they are being sold for a low price from an unknown individual.

Quarantining new animals is essential for risk reduction in relation to infectious disease. A quarantine period of at least 90 days is recommended for new snake acquisitions and animals that have been to reptile swaps, although some new animals may have to be quarantined for longer periods of time. Ideally, quarantined animals should be housed in a building that is separate from the main collection. A true quarantine, which is rarely done, takes place when all new animals enter the facility on the same day and leave on the same day, 90 days later. For IBD evaluation, blood is the easiest diagnostic sample to collect in snakes, and blood smears can be examined for the presence of IBD inclusions in blood cells. If snake-keepers have the financial resources, biopsy samples can be obtained from a snake's tonsils, liver, and kidneys. Collection of biopsy samples is not practical in all situations, but antemortem diagnostic testing improves the owner's ability to identify an infected animal. In the end, the cost of recommended quarantine programs and the use of diagnostic testing depend on either the owner's financial situation or the importance they attach to such preventative medicine programs.

Finally, snakes showing clinical signs of illness or anorexia and weight loss should never be added to an established collection. Only mite-free animals with a good appetite and excellent body condition should be added after the quarantine period is over. Once mites infest a large snake collection, they may be impossible to totally eliminate.

Managing a Collection

Seeing a single lone IBD-positive snake in a collection is uncommon. Typically, where there is one case of IBD there are others. As mentioned before, subclinical cases of IBD are common. So what should be done in a situation where IBD has been identified in a snake collection? In extreme cases, some people have depopulated entire collections of susceptible snakes where IBD had been diagnosed and where a large number of the snakes shared the same room or had been in contact with one another. Other owners will be in denial despite evidence that IBD exists in their collection. Snake owners in denial often decide to conduct business as usual. This wide range of owner responses is typical for any type of animal-breeding operation when a potential infectious disease has been identified.

What has made a difference in controlling and managing infectious disease outbreaks in domestic animals is 2-fold. First, there is the ability to develop very sensitive and specific diagnostic tests for determining exposure to a specific pathogen. Currently, demonstration of inclusions using H&E staining is the only diagnostic method being used to identify cases of IBD. The sensitivity and specificity of this staining technique for IBD have not been established. Second, there is the development of vaccines that can protect animals from infection and disease. Unfortunately, there is no vaccine available for protection against IBD or any effective treatment. Vaccine research and development are extremely costly, and the odds that the money will become available for an IBD project are extremely low.

There are still options available to both veterinarians and owners to determine the IBD status of the animals they are presented with or own. Biopsy samples and blood smears can be obtained from exposed animals to screen for inclusions. If it is financially impossible to obtain samples from all animals within a collection, then select a subset for evaluation. Snakes showing signs of IBD should be immediately removed from the main collection and placed in a separate room, or submitted for a microscopic and/or necropsy evaluation. Cages of ill or dead animals should be disinfected with bleach and allowed to dry in the sunlight for a few days. Household bleach is the best overall disinfectant and must come into contact with cleaned surfaces. Although it will not kill every pathogen known to snakes and other reptiles, it is highly recommended.

A question that has been asked many times is whether the agent causing IBD can be transmitted to neonates of either live-bearing or egg-laying snakes. We have very little information to determine whether vertical transmission of this disease is possible. There are anecdotal reports that recently hatched ball pythons were diagnosed with IBD, but microscopic slides were not forwarded to the authors for substantiation. One of the authors (ERJ) received a series of neonate boa constrictors that had an IBD-positive mother, but inclusions of the disease were not found in the offspring of that animal. The problem with trying to determine if vertical transmission of IBD is possible is related to a lack of appropriate diagnostic tests; molecular-screening tools that typically have a high degree of sensitivity and specificity are currently not available. The conservative viewpoint is to assume that the agent can be transmitted to offspring; therefore, neonates from known IBD-positive females should not be kept, sold, or given away.

Present and Future Research Needs

A study by Wozniak and coworkers demonstrated that inclusion bodies in IBD are composed of an

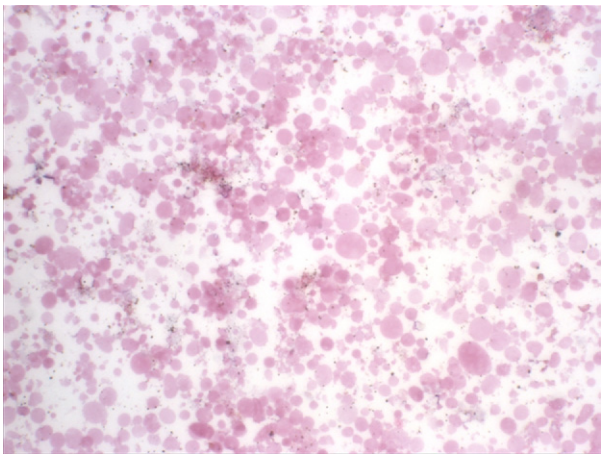

Figure 18. Boa constrictor. A semipurified preparation of inclusions obtained from the liver of a boa constrictor with IBD. H&E staining.

antigenically unique 68-kd protein.[5] Monoclonal antibodies (MAB) against IBDP have been produced that recognize an IBDP band in a western blot and IBDP antigen in frozen tissue sections using immunohistochemical (IHC) staining. Unfortunately, the original anti-IBD MAB was lost. Recently, we have isolated inclusions (Fig 18) from a case of IBD and, using hybridoma technology, have produced a new anti-IBDP MAB that stains inclusions in paraffin-embedded tissues (Fig 19, A and B). This MAB is going through rigid testing and validation. Staining using IHC could be a very helpful first step in developing an immunologic-based or molecular-based diagnostic method that will allow us to identify infected snakes that are in an early stage of infection. This MAB will also be helpful in distinguishing inclusions consisting of IBDP from those inclusions resembling but distinct from IBD inclusions.[15]

It is unknown whether all IBD cases in different snake species consist of exactly the same protein or if variability occurs that is species dependent. Western blot is another practical test that can be developed for making an antemortem diagnosis. Using Western blot technology, inclusion protein in small amounts obtained from biopsied tissue or isolated peripheral white blood cells from a blood sample can be tested. Studies to evaluate the sensitivity and specificity of the diagnostic testing methods described above are planned.

Some of the authors' current work centers on a better understanding of the IBD protein composition and the development of a test to identify its presence. Identifying the amino acid sequence of IBDP will be a critical step in eventually understanding the pathogenesis of IBD. Once the sequencing data for IBDP are obtained, bioinformatic analysis can be used to predict its origin, structure, and biochemical properties. Through peptide synthesis, the antigenic region of the IBDP can be produced and used as a better source of antigen for developing an enzyme-linked immunosorbent assay or other immuno-based molecular diagnostic tests. Concurrently, retroviruses isolated from tissue of IBD-infected snakes[4] have been recovered and are growing in culture. Once the virus is purified and sequenced, specific primers against the viruses will be made and used in a polymerase chain reaction test. The use of a polymerase chain reaction–based test will be invaluable in determining if there is consistent association of this retrovirus and IBD.

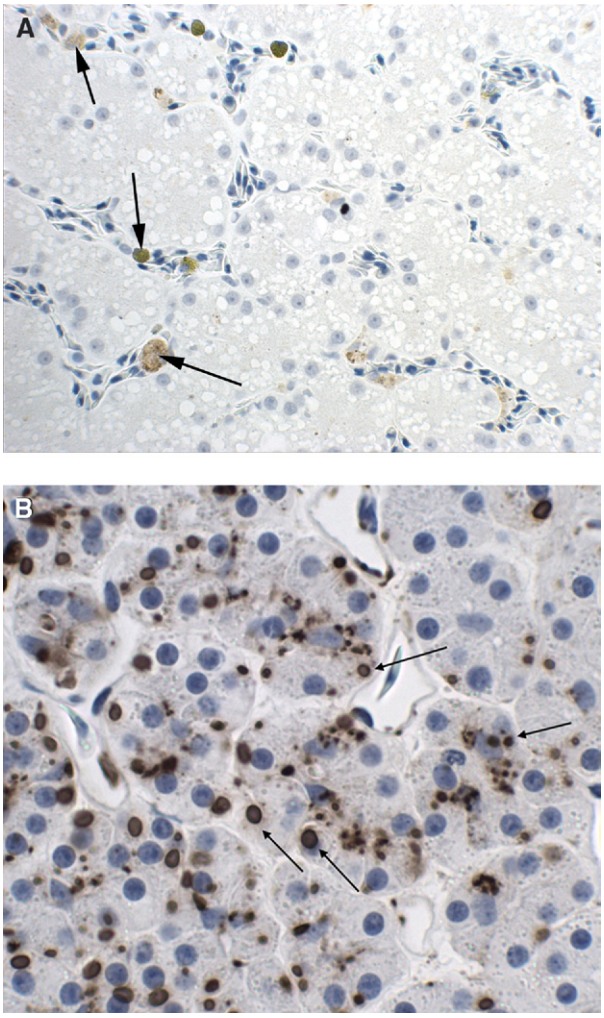

Figure 19. (A) Boa constrictor. Liver. Negative-control staining. The MAB prepared against IBD inclusion protein was excluded from the IHC staining protocol. Several heterophils with light nonspecific staining can be seen (arrows). Immunoperoxidase stain. (B) Boidae. Liver. Positive-control staining. The MAB prepared against IBD inclusion protein was included in the immunohistochemical staining protocol. Positive staining of inclusions can be seen (arrows). Immunoperoxidase stain.

If there is any hope or desire for IBD to be managed in breeding operations of snakes sold for the pet trade, the need for such molecular-based tests is clear. The main problem scientists have regarding IBD research is that research dollars needed to perform the investigations have been extremely limited. Although several reptile expositions and a number of private individuals have made contributions to this work, the total number of dollars received has been inappropriate for the studies needed to have a significant impact on determining the cause of IBD and developing timely, cost-effective, scientifically validated diagnostic tests.

Concluding Remarks

IBD is one of the few worldwide diseases of captive snakes. The ease at which snakes can be shipped around the world is probably responsible for its spread. Because many IBD-positive snakes may be subclinically infected, infected snakes considered healthy have been sold. It is possible that subclinical IBD infections can persist for long periods. It is unknown where this disease originated because there are no reports of IBD in wild populations. A sensitive and specific blood-based immunodiagnostic and/or molecular test is needed to screen blood samples of both captive and wild populations of boid snakes. Maintaining healthy and disease-free animals should be the primary responsibility for all who keep, breed, and intend to release captive bred and rehabilitated animals back to the wild.

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