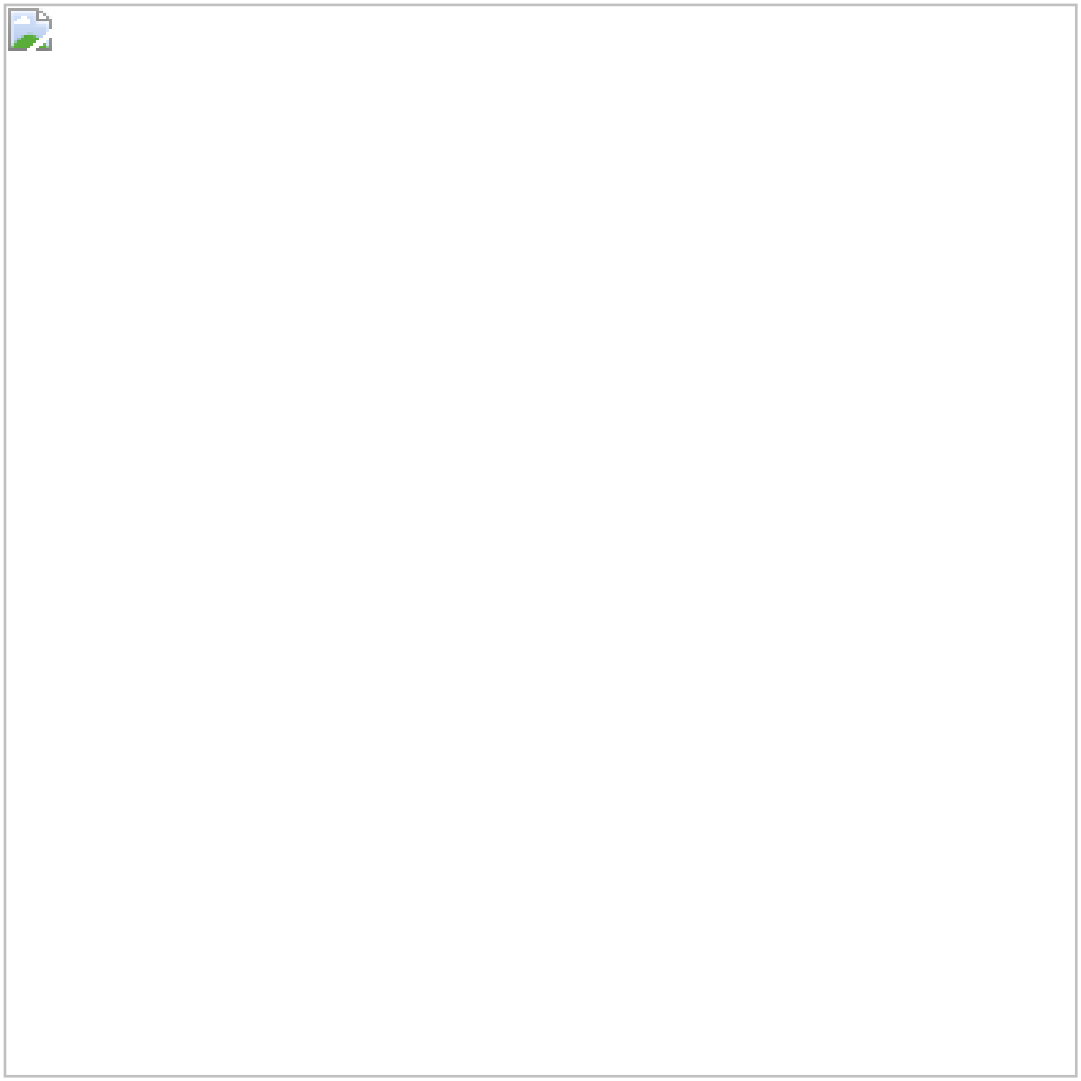

INDICATIONS OF A VIRAL ETIOLOGY FOR MARBLE SPLEEN DISEASE IN PHEASANTS 1

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INDICATIONS OF A VIRAL ETIOLOGY FOR MARBLE SPLEEN DISEASE IN PHEASANTS[1]

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Abstract: Evidence that marble spleen disease of pheasants (*Phasianus colchicus*) has a viral etiology was obtained from field cases using the following criteria: Intranuclear inclusion bodies in cells of the spleen, lungs, liver, and bone marrow; the presence of antigen in spleens of infected pheasants as detected by the agar gel precipitin test; demonstration of virus particles in the splenic intranuclear inclusions by the electron microscope; and the presence of specific fluorescence in spleen cells as shown by direct fluorescent antibody staining. Every case of marble spleen disease examined had all of these findings. Attempts to isolate a virus in cell culture using chicken and pheasant embryo fibroblasts and chick kidney cells were unsuccessful.

INTRODUCTION

Previous investigators[1,2,9,10,11] have established that marble spleen disease (MSD) is a peracute, fatal disease of pen reared ring-necked pheasants (*Phasianus colchicus*) in which splenic necrosis and pulmonary edema are prominent features, and the demonstration of virus particles in splenic reticulum cell nuclei (spleen cells) of pheasants with MSD using electron microscopy suggested that the causative agent might be a virus.[2,11]

This study was undertaken to further substantiate the hypothesis of a viral etiology for MSD. Microscopic lesions of MSD were correlated with: 1) Presence of antigen in spleens of MSD-infected pheasants detected by agar gel precipitin (AGP) tests; 2) presence of virus particles in spleen cells detected by electron microscopy; and 3) presence of specific fluorescence in spleen cells demonstrated by direct fluorescent antibody staining. In addition, attempts to isolate a virus using pheasant embryo fibroblasts, chicken embryo fibroblasts, and chick kidney cells were made.

MATERIALS AND METHODS

Pathology

Fifteen field cases of suspected MSD in pen reared pheasants were obtained from Connecticut, Massachusetts, and Pennsylvania. Viscera were examined grossly and microscopically to establish the diagnosis. Portions of spleen, bone marrow, intestine, lung, liver, heart, brain, kidney, skin, and salivary glands were fixed in 10% formalin, sectioned at 5 μ, and stained with hematoxylin and eosin.

Cell Cultures

Chicken embryo fibroblasts (CEF), pheasant embryo fibroplasts (PEF), and chick kidney cells (CKC) were inoculated in six trials with infected spleens obtained from field cases and quick frozen in the field. Inocula consisted of 0.1 ml of spleen homogenate diluted 1:1 with Eagle's minimal essential medium (MEM) (10% tryptose phosphate broth and 1% calf serum). For incoulation, spleens were homogenized in a Ten Broeck grinder and then either sonicated

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at 40 watts for 30 sec or inoculated as homogenates. Cultures were inoculated and absorbed for 1 h and then maintained for three serial passages and observed daily for cytopathic effect (CEP). Also, monolayers and their supernatant fluids were tested for antigen by the AGP test at various times after each passage.

Agar Gel Precipitin Tests

Presence of precipitinogen in the spleens of MSD-infected pheasants was detected using the AGP test described by Jakowski and Wyand.[5] Tissues tested for antigen were prepared from skin, lung, liver, kidney, and spleens by homogenization with a Ten Broeck grinder and then freeze-thawing 4 times. Antisera were obtained from pheasants in flocks exposed to MSD.

Electron Microscopy

Small pieces of spleen were carefully excised with a razor blade and placed in 4% phosphate buffered glutaraldehyde (pH 7.4) at 4 C for 2 hours, after which they were rinsed in two changes of phosphate buffer. Tissues were post-fixed in 2% phosphate buffered osmium (pH 7.4) and dehydrated in a graded series of ethanol, followed by propylene oxide. Pieces of spleen were then embedded in Luft's Epon Araldite, sectioned on an LKB Ultromicrotome-3, stained with lead citrate, followed by uranyl acetate, and examined with a Jelco T6S electron microscope.

Fluorescent Antibody Staining

The globulin fraction of two pheasant sera having titers of 1:8 by the AGP test were obtained using Chase's[3] modification of Kekwick's procedure.[7]

A protein determination was performed by the method of Lowry *et al.*[8] Conjugation of the globulin with fluorescein isothiocyanate (FITC) was done by the method of Holbrow and Johnson[4] with modifications. Six milliliters of globulin solution were chilled at 4 C and 1.0 ml of carbonate-bicarbonate buffer (pH 9.0) at 4 C was added. FITC, in a ratio of 20:1 protein to FITC, was added in powder form and stirred gently with a magnetic stirrer for 18 h at 4 C. Following clearing by centrifugation, the preparation was dialyzed against several changes of phosphate buffered saline (PBS) (pH 7.2) for 36 h. Excess fluorochrome was removed on a G-25 Sephadex column (Pharmacia Fine Chemicals, Piscataway, New Jersey), with PBS used as an eluant. The dilute conjugate was then placed in an Amicon concentration unit (Amicon Corp., Lexington, Massachusetts) with a PM-10 membrane under 14 pounds of nitrogen pressure and concentrated to the original volume of 6.0 ml.

Fluorescent staining of splenic imprints and frozen sections of spleen, lung, and liver was done by a modified Kawamura[6] method. Sections were mounted on clean microscope slides and dried at room temperature and then fixed in acetone at —20 C for 40 min. Slides were removed from the acetone and placed in PBS for 1 min to remove the acetone, and then blotted to remove excess buffer. The conjugate was applied to the tissue and incubated in a humid athosphere for 45 min at 37 C, followed by washing 5 times with PBS for 30 min with a final brief rinse in distilled water. Coverslips were mounted using PBS and glycerine in a 9:1 ratio. Splenic imprints were counterstained with equal parts of Evans Blue and PBS following conjugate staining and rinsing. To prove the specificty of fluorescence, blocking serums, consisting of purified pheasant globulin with AGP antibody and pheasant serum with AGP antibody, were used. Normal pheasant serum, without AGP antibody, was used to determine if some serum component might non-specifically block fluorescence.

RESULTS

Gross Lesions

Of 15 birds examined, 11 had enlarged mottled spleens, and diffusely hemorrhagic congested lungs (Table 1). These findings have been reported previously as characteristic of MSD.[1,2,9,10,11] No other gross lesions were observed.

TABLE 1. Correlation of Results Indicating a Viral Etiology for Marble Spleen Disease (MSD).

Findings	Number examined	Number positive	Number negative*
Gross lesions of marble spleen diseases	15	11	4
Intranuclear inclusions in spleen cells	15	11	4
Virus particles in spleen cell nuclei	12	8	4
Spleen cellular fluorescence	14	10	4
Splenic antigen by AGP testing	15	11	4

* The cases in the negative column are the same four pheasants. These birds died from undetermined causes and are included in the column "Number examined".

Microscopic Lesions

Microscopic lesions included splenitis, splenic necrosis, and amyloidosis. Many splenic reticulum cells contained typical intranuclear inclusions.[1,2,9,10,11] Tertiary bronchi and air spaces contained erythrocytes and fibrin and disseminated foci of necrosis were seen in the lung parenchyma. Intranuclear inclusions were observed infrequently in the lungs. Additional microscopic findings consisted of intranuclear inclusions in the liver and bone marrow cells. Although intranuclear inclusions were numerous in the liver, we were unable to determine by light microscopy whether they occurred in hepatocytes or sinusoidal reticuloendothelial cells. Carlson *et al.*,[2] however, reported inclusions to be in Kupffer cells.

Cell Cultures

No CPE was observed in six trials using CEF, PEF, and CKC cultures passaged serially 3 times and inoculated with homogenized and homogenized–sonicated infected spleens from field cases. Cell monolayers and their supernatant fluids were negative for antigen by the AGP test in all three passages.

Agar Gel Precipitin Tests

Antigen was detected in the spleens of 11 cases of histologically confirmed MSD. No antigen was demonstrated in lung, kidney, liver, and skin. The spleens of four pheasants in which lesions of MSD were absent were also negative for antigen.

Electron Microscopy

Twelve spleens, of which four were from birds without gross or microscopic lesions of MSD, were examined. Splenic intranuclear inclusions contained virus particles approximately 70 nm in diameter in the cytoplasm as well as in the nucleus (Fig. 1), and these were seen in each of the eight cases with gross and microscopic lesions (Table 1). The four cases without lesions had no virus particles or splenic inclusions. No enveloped virus particles were observed. The majority of virus particles seen with electron microscopy in splenic inclusions were present in the central portion of the inclusion, corresponding to the area that stained eosinophilic with hematoxylin and eosin (Fig. 2).

Fluorescent Antibody Staining

Lungs, liver, and spleen from six pheasants with MSD were examined by the frozen section technique. Cells with specific fluorescence were seen in the spleens only (Fig. 3). Spleens and livers from four pheasants with MSD were examined by the imprint technique and were counterstained with Evans Blue. In these four cases cells of the liver, as well as the

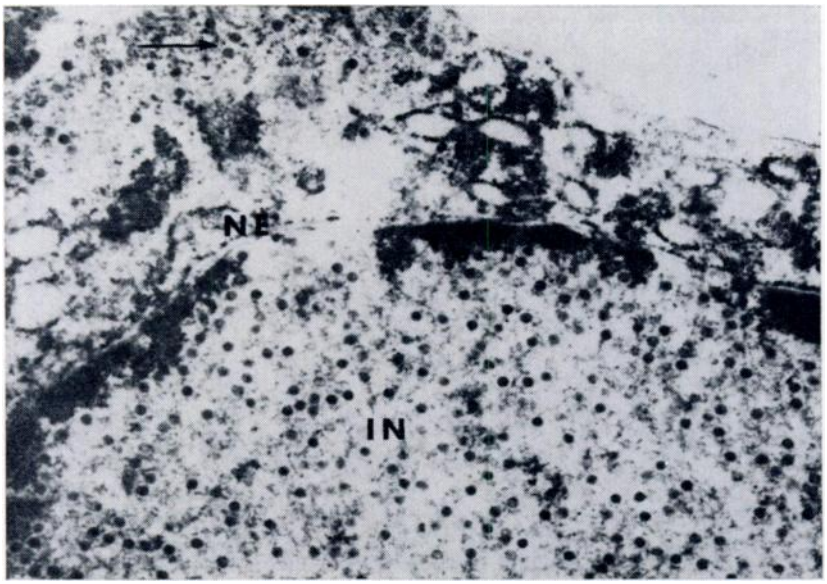


FIGURE 1. Electron micrograph of spleen. Virus particles are present in the intranuclear inclusion (IN) and the cytoplasm (arrow) of a cell. The nuclear envelope (NE) appears to be disintegrating (X 21,000).

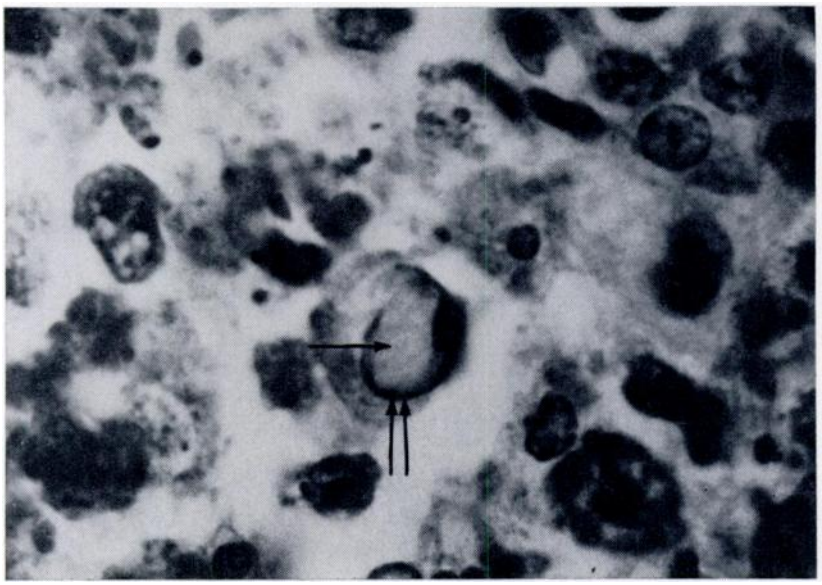
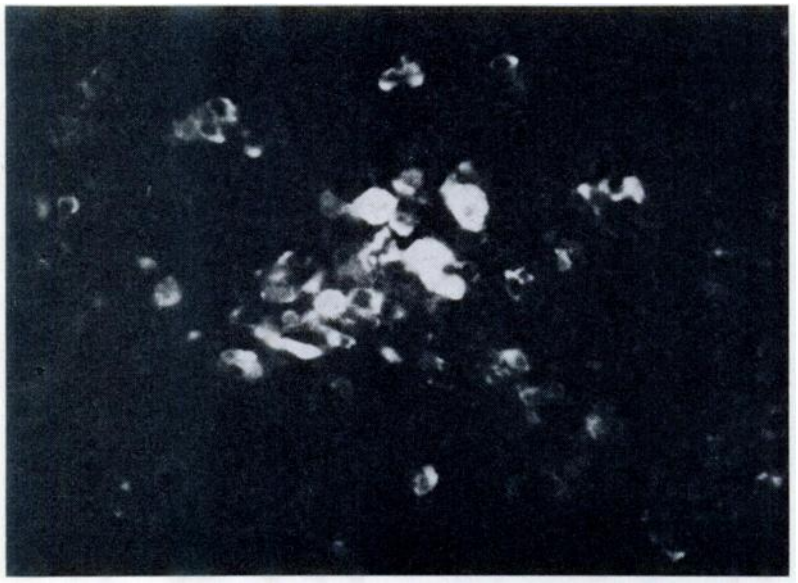


FIGURE 2. Intranuclear inclusion in a spleen cell. The arrow indicates the eosinophilic inclusion which contains large numbers of virus particles. Double arrows indicate a basophilic nuclear rim (Hematoxylin and eosin: X 1,650).



FIGRE 3. Spleen. Fluorescent cells in a frozen section from a pheasant with MSD. Direct fluorescent antibody staining (X 200).

FIGURE 4. Spleen. Cell nucleus with peripheral fluorescence. Imprint preparation counterstained with Evans Blue. Direct fluorescent antibody staining (X 660).

spleen, had specific fluorescence. Blocking reactions eliminated most of the fluorescence. Normal pheasant serum (without AGP antibody) applied prior to conjugated serum did not reduce specific fluorescence.

Determination of the location of cellular fluorescence was possible by alternating light and dark field microscopy on the same field of cells. Using this procedure it was determined that fluorescence is primarily nuclear and is most intense along the inner margin of the nuclear envelope. (Fig. 4) This inner nuclear rim stained basophilic (Fig 2) with hematoxylin and eosin and did not contain any significant number of virions as evidenced by electron microscopy (Fig. 1). A few cells had diffuse stippled cytoplasmic fluorescence. The four cases without lesions were negative for specific cellular fluorescence.

DISCUSSION

The results presented here indicate that a virus is very likely the etiologic agent of MSD. Indirect evidence for a viral etiology is based on the following: 1) Intranuclear inclusions in cells of bone marrow, spleen, lung, and liver demonstrated by light microscopy; 2) Antigen in spleens of MSD-infected pheasants detected by AGP tests; 3) Specific cellular fluorescence in spleens and livers determined by direct fluorescent staining; and 4) The presence of virus particles in splenic intranuclear inclusions shown by electron microscopy. Each of these findings occurred in every case of MSD examined. None of the above were found in any material from four pheasants without MSD. Thus, a correlation has been shown between the presence of this disease and the following: Splenic antigen, spleen and liver cell fluorescence, intranuclear inclusions, and virus particles in splenic cell intranuclear inclusions.

Virus particles seen in spleen cells may be in the adenovirus group because of their predominanly nuclear location, 70 nm diameter, and absence of envelope. Virus particles seen in the cytoplasm were always dispersed in proximity to the nucleus and were not observed in specific locations or quantities that would indicate replication in the cytoplasm. Therefore, virus observed in the cytoplasm may be the result of passage through a disintegrating nuclear envelope.

Microscopic examination did not reveal lesions in the skin, brain, kidney, intestine, salivary glands, and heart. Lesions were restricted to lung, liver, spleen, and bone marrow.

Attempts to isolate the virus in cell culture were not successful, using CPE and AGP detectable antigen as criteria of infection. Explanations for these observations may be that either the virus replicates without manifesting CPE or antigen production, or the proper cell type needed for replication was not used.

The location of fluorescence peripherally along the nuclear envelope would indicate that antibody from serum of pheasants exposed to MSD has specificity to some viral protein that has marginated, as does the nuclear chromatin. The fact that there is no fluorescence in the region of the inclusion containing large numbers of virus particles may indicate that the virus itself is not the antigen which gave rise to antibody which was conjugated for these studies. It is also possible that the antigen demonstrated by fluorescence is an altered cellular protein due to viral infection and has stimulated antibody production upon escape from cells damaged by the virus.

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