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EXPERIMENTAL REPRODUCTION AND ANTIBODY INHIBITION OF MARBLE SPLEEN DISEASE OF PHEASANTS[1]

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Abstract: An extract of spleens from three pheasants affected with marble spleen disease was used as an intravenous inoculum to transmit the disease to pen reared pheasants (*Phasianus colchicus* x *Phasianus torquatus*). The disease was prevented by specific convalescent pheasant antiserum and by antiserum from turkeys that had recovered from hemorrhagic enteritis of turkeys. The causative agent of the disease passed through 0.22 μ filters, resisted chloroform and retained its precipitin antigen quality after propagation by bird passage. Filterability, chloroform resistance, antigenic characteristics and *in vivo* response to antibody strongly indicate that the causative agent of marble spleen disease is a virus very similar to the virus which causes hemorrhagic enteritis of turkeys.

INTRODUCTION

In the U.S.A., marble spleen disease of pheasants apparently was observed first about 17 or 18 years ago and was transmitted at that time to one experimental bird.[7] Since then, the gross and microscopic lesions of the disease have been described, a precipitin antigen extracted from acutely affected spleens has reacted with convalescent antibody, fluorescent antibody has reacted with intrasplenic antigen, a virus thought to be causatively associated with the disease has been demonstrated by electron microscopy and a viral etiology has been postulated. All of these findings recently have been reviewed or reported by Iltis and Wyand.[5]

The purpose of this paper is to report the experimental transmission of marble spleen disease of pheasants, to present further evidence of its viral etiology, to report the effect of convalescent antiserum administration on the disease and to present additional evidence that the causative agent of marble spleen disease of pheasant and the causative virus of hemorrhagic enteritis of turkeys are closely related.

MATERIALS AND METHODS

Experimental Birds

All pheasants were ring-necked x Korean pheasant crosses, All turkeys were Medium White.

Precipitin Test

All serum samples were tested for antibody and all spleens were tested for antigen associated with marble spleen disease of pheasants[6] by agar gel diffusion according to the method of Domeruth et al.[2] This method closely resembles the method of Jakowski et al.[6]

All pheasants were tested for antibody to marble spleen disease prior to their experimental use.

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Transmission Studies

Experiment 1

Three spleens were obtained from pheasants which had died of marble spleen disease in 1970 and 1971. All spleens were kept frozen at —22 C or lower from 1970 to time of experiment. The spleens were minced separately, diluted to 50% in aqueous solutions of 0.85% NaCl, frozen, vigorously shaken, and centrifuged at 2000 G for 5 min at room temperature.[2] The supernatant fluids obtained by this treatment were further diluted to 10^{-1} in 0.85% of aqueous NaCl and 0.2 ml of each sample was injected intravenously into each of three 1-year-old and three 12-week-old pheasants. Thus, six pheasants were injected with each of the splenic extracts. An additional six birds were kept as uninoculated controls. Two birds from each group of six were necropsied 4 days after inoculation (as is our practice when studying hemorrhagic enteritis of turkeys[2]), spleens were observed for marbling, weighed, tested for presence of antigen,[2,6] and sectioned and stained with hematoxylin and eosin for histopathological studies. Unused spleen was retained to prepare an inoculum for Expts. 2 and 3. Lungs were also observed for presence of hemorrhage and congestion. The remaining birds in the inoculated groups were held for 2 weeks to permit development of convalescent antiserum which was pooled and used in Expts. 2 and 3. The pooled convalescent serum from these pheasants was positive for antibody associated with marble spleen disease (see MATERIALS AND METHODS, Precipitin Test).

Experiment 2a

One-half of each spleen collected in Exp. 1 was minced and pooled to produce splenic extract to use as inocula for birds in all of the following experiments. Extract preparation was performed as previously described in Exp. 1. All pheasants used were 1 year old.

Group 1. Four pheasants were each inoculated orally with 0.2 ml of inoculum.

Group 2. Four pheasants received 0.2 ml of inoculum orally and simultaneously 1.0 ml of convalescent marble spleen disease antiserum (source described in Exp. 1) was injected subcutaneously in the neck region.

Group 3. Four pheasants were inoculated orally with 0.2 ml of pooled splenic extract and each bird also received 1.0 ml of convalescent turkey hemorrhagic enteritis antiserum subcutaneously in the neck. This antiserum is further described in the publication by Domermuth et al.[2]

Group 4. Four pheasants were inoculated orally with 0.2 ml of pooled splenic extract which was first centrifuged at 10,000 G for 10 min and filtered successively through two 0.22 μ cellulose filters.

Group 5. Four pheasants were inoculated orally with 0.2 ml of pooled splenic extract that had been vigorously shaken with equal volumes of chloroform at room temperature (25 C) followed by centrifugation (20 C) at 10,000 G for 10 min. The treated supernatant splenic extract was removed and used as inoculum. This method of chloroform treatment is similar to the method of Feldman and Wang.[1]

Group 6. Four pheasants received no inoculum and were used as negative controls.

Birds in all groups were necropsied on day 6. Spleens were weighed, observed for marbling, and tested for antigen. The lungs were examined for edema.

Experiment 2b

Groups 1, 3, 4, 5 and 6 of Exp. 2a were replicated using three pheasants per group and another aliquot of the inoculum described in Exp. 2a.

Experiment 3

Exp. 2a was duplicated in 6-week-old turkey poults.

RESULTS

Precipitin Test

No pheasant or turkey poult had detectible marble spleen disease antibody prior to experimental use.

Transmission Studies

Experiment 1

All six spleens of inoculated pheasants necropsied 4 days post-inoculation were enlarged and marbled, averaged 1.67 g in weight, and were positive for antigen by the precipitin test. Spleens of the two uninocluated control pheasants were not enlarged, were negative for precipitin antigen and averaged 0.68 g in weight.

Microscopic lesions observed in the sections of enlarged and marbled spleens resembled those which have recently been described and reviewed by Iltis and Wyand.[5] No lesions were observed in sections of spleens of uninoculated control birds.

Serum samples of four of the twelve remaining inoculated pheasants were strongly positive, four were weakly positive, and four were negative for marble spleen disease precipitin antibody.

Experiment 2a

The most significant observation, perhaps, was that one inoculated pheasant died of what appeared to be typical marble spleen disease. Gross lesions included an enlarged mottled spleen and diffusely hemorrhagic congested lungs.

Other significant data, summarized in Table 1, include the observations that convalescent antiserum from both marble spleen disease-affected pheasants and from hemorrhagic enteritis-affected turkeys prevented splenic enlargement and mottling in pheasants inoculated with infectious splenic extract. The findings that the splenic extract remained infectious after multiple filtration (0.22 μm) and after chloroform treatment are also significant.

Experiment 2b

Results from this experiment were very similar to those from Exp. 2a except that the spleens of the three pheasants that received untreated splenic extract (no chloroform treatment or filtration) were not as enlarged as those from the four similar pheasants in Exp. 2a, nor did they contain detectible antigen (Table 1).

TABLE 1. Pheasant Inoculation Results — Experiments 2a and 2b.

Group	Regime	No. Per Group	No. Dead	Spleens + for Antigen	Average Grams per Spleen
1	Oral splenic extract[1]	4 (3)[4]	1	4 (0)	2.25 (1.2)
2	Oral splenic extract plus pheasant antiserum[2]	4	0	0	0.82
3	Oral splenic extract plus turkey antiserum	4 (3)	0	0 (0)	0.73 (1.07)
4	Oral filtered (0.22 μ) splenic extract	4 (3)	0	4 (2)	2.1 (2.3)
5	Oral chloroform treated splenic extract	4 (3)	0	4 (2)	2.3 (1.53)
6	No inoculum	4 (3)	0	0 (0)	0.66 (0.75)

[1] Oral splenic extract, 0.2 ml per bird.
[2] Convalescent marble spleen disease antiserum, 1.0 ml under skin of neck.
[3] Convalescent turkey hemorrhagic enteritis antiserum, 1.0 ml under skin of neck.
[4] Numbers in parentheses are from experiment 2b.

Experiment 3

Data from inoculation of turkey poults were essentially similar to those from pheasants in Exp. 2a, except that the spleen was the only organ grossly involved. These data are presented in Table 2. Perhaps of greatest significance is that the virus was experimentally propagated in turkeys.

TABLE 2. Turkey Inoculation Results — Experiment 3.

Group	Regime	No. Per Group	No. Dead	Spleens + for Antigen	Average Grams spleen
1	Oral splenic extract[1]	6	0	5	3.7
2	Oral splenic extract plus pheasant antiserum[2]	6	0	0	2.1
3	Oral splenic extract plus turkey antiserum[3]	6	0	0	2.26
4	Oral filtered (0.22 μ) splenic extract	6	0	4	3.72
5	Oral chloroform treated splenic extract	6	0	5	3.6
6	No inoculum	6	0	0	2.04

[1] Oral splenic extract, 0.2 ml per bird.

[2] Convalescent marble spleen disease antiserum, 1.0 ml under skin of neck.

[3] Convalescent turkey hemorrhagic enteritis antiserum, 1.0 ml under skin of neck.

DISCUSSION

It appears that this is the first report of replicated experimental transmission of marble spleen disease of pheasant, of the suppression of lesions of the disease by injection of antibody, of evidence of viral etiology supported by filtration and resistance to chloroform and of propagation of the virus in turkey poults.[4] The authors believe that their strict adherence to the experimental rationale gained by working with hemorrhagic enteritis of turkeys,[2,3] which appears to be caused by a similar adenovirus-like agent,[1,8] contributed in large measure to the successful *in vivo* studies presented in this report. The most important principles essential for successful *in vivo* propagation of both agents seem to be:

(1) Attempt propagation only in birds that do not have antibody to either disease. If parent birds have antibody or have had clinical disease, the young birds being raised for experimental use must be raised in complete isolation from the parent stock on premises where the disease has not occurred.

(2) Attempt infection only in birds that have reached the age at which the natural field disease generally occurs. This is about 5 or 6 weeks of age for turkeys and 10 or 12 weeks for pheasants.

(3) If the infectious splenic extract is inoculated intravenously, necropsy should be done 3-4 days later. If inoculation is via the oral (or rectal) route, necropsy should be done 6-7 days later. Maximal lesions occur at these times in hemor-

[4] A paper on this topic by Iltis, J. P., R. M. Jakowski and D. S. Wyand is scheduled to appear in the January 1975 issue of the Am. J. vet. Res.

rhagic enteritis.[2,3] Spleens seem to remain enlarged, infectious and antigenic for only a day or two in hemorrhagic enteritis. It therefore seems likely that this also may prove to be true for marble spleen disease.

The fact that injection of convalescent antibody will prevent lesions of marble spleen disease in experimentally infected birds strongly suggests that a similar practice will stop or reduce the spread of the disease in affected field flocks, at least for 3 or 4 weeks post injection.

The production of smaller spleens with no detectible antigen in the second attempt to produce the disease with untreated splenic extract (Exp. 2b, Group 1) could have been due to insufficient breakdown of splenic tissue to permit optimal liberation of virus; however, the slight enlargement of the spleens indicates that they may have been in an earlier stage of infection, infected with fewer virions, or possibly infected with an increased ratio of incomplete to complete virions present in the untreated inoculum.

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