STUDIES ON THE CAUSATIVE AGENT OF AN EPIZOOTIC AMONGST MANX SHEARWATERS (*PUFFINUS P. PUFFINUS*)

By M. G. P. STOKER and J. A. R. MILES*

Department of Pathology, University of Cambridge

(With Plate 8)

INTRODUCTION

A widespread and highly fatal epizootic amongst juvenile Manx shearwaters (*Puffinus p. puffinus*) on the island of Skomer was first reported by Surrey Dane (1948) and more recently by Surrey Dane, Miles & Stoker (1953). The disease occurs each year in September and affects the juvenile birds soon after they have been deserted by their parents about 2 months after hatching, but before they disappear to sea. The affected birds develop large blisters on the webs (see Pl. 8) and occasionally conjunctivitis. In the years 1947–50 up to 70 % of the juveniles were involved depending on the locality and the year of study, and it was thought that most of the affected birds subsequently died. Post-mortem examination revealed no macroscopic abnormality except for the blisters on the webs and, in some birds, patches of consolidation in the lungs. Histologically there was much inflammation round the blisters, and some of the cells of the epidermis showed cytoplasmic eosinophilic inclusions. On culture, the blister fluid generally yielded *Staphylococcus aureus* or a mixed bacterial flora, but the blood was sterile.

In a preliminary communication Miles & Stoker (1948) reported the isolation of a virus from the blood of a sick shearwater in the 1947 epizootic. This paper describes the laboratory studies which have been carried out on this agent and on further strains which have been isolated subsequently.

ISOLATION OF VIRUS

Bacteriologically sterile blood taken in 1947 from a moribund shearwater with large blisters was stored overnight at $+4^\circ$ C. A suspension of the clot was then inoculated on to the chorio-allantoic membrane (c.a. membrane) of 10-, 12- and 13-day-old fertile white Leghorn hens eggs. After incubation for 48 hr. at 36° C. the membranes showed a few round opacities which were vaguely suggestive of virus lesions. Membranes left for 72 hr. were diffusely thickened and showed similar lesions. The membranes were bacteriologically sterile, and sections showed ectodermal proliferation and intracytoplasmic eosinophilic inclusions in the ectodermal cells, which were reproduced in subsequent passages.

A further strain was isolated in the following year (1948) from pooled blister fluid from two sick birds. This was diluted tenfold in broth and filtered through

13-2

a 'Gradocol' membrane with an average pore diameter (A.P.D.) of 750 mμ before inoculation of the C.A. membrane as before. Another strain was isolated in 1948 from mites (*Trombicula autumnalis* Shaw) collected from shearwaters, and a fourth strain was isolated from blister fluid obtained in the 1949 epizootic. The 1947 strain in particular was used for inoculation of a variety of birds and mammals as well as fertile eggs. The results of these experiments showed susceptibility of fertile hens' eggs and of certain birds, but no evidence of infection was found in any of the mammals investigated.

GROWTH OF THE VIRUS IN FERTILE HENS' EGGS

The 1947 strain was maintained for forty-two passages in fertile eggs by inoculation of the C.A. membrane. 0·2 ml. quantities of bacteriologically sterile broth suspensions of membranes from previous passages, suitably diluted, were inoculated on to the C.A. membranes of fertile hens' eggs previously incubated for 10 or 11 days at 38° C. After incubation for 48 or 72 hr. at 36° C. the inoculated areas were removed and examined microscopically. Suitable pieces were chosen for histological examination; other pieces were cultured aerobically and sometimes anaerobically on blood agar.

The macroscopic appearances of the inoculated membranes during the first twelve passages were variable and, although suggestive of a virus infection, they were not sufficiently characteristic to be used for identification. Usually generalized haziness and oedema were observed and occasionally small round opacities were present, but it was difficult to distinguish these from non-specific lesions. No elementary bodies suggestive of virus particles were seen in smears made from these membranes when stained by a variety of techniques. Histologically, however, the inoculated membranes all showed the following characteristic change. There was hyperplasia of the ectodermal layer with a variable amount of meso-dermal reaction underneath. The hyperplastic ectodermal cells were swollen and the nuclei very variable in size. Some of the large ectodermal cells contained one or more homogeneous eosinophilic cytoplasmic inclusions, round, oval or elongated and varying from 2 to 10μ in their largest diameter (see Pl. 8, fig. 2). In eggs harvested more than 48 hr. after inoculation, there was usually some superficial ectodermal necrosis in the thickened areas, and the inclusions were generally larger but not more numerous. All these changes were readily seen in sections stained with haematoxylin and eosin, but the inclusions were more obvious when stained by Mann's method (Van Rooyen & Rhodes, 1948).

Membranes which had been inoculated with broth alone showed some areas of non-specific, mainly mesodermal, cellular infiltration, and fragments of red cells were sometimes visible as orange red inclusions in the cytoplasm of macrophages, but it was not difficult to distinguish these from the specific inclusions which were present in the cells of the ectoderm and were purple-red in colour when stained by Mann's method. Passages of uninfected membranes were also carried out, but the specific histological appearances seen in infected membranes were never found. These specific histological changes, and particularly the characteristic inclusion bodies, were therefore taken as the main criterion of infection.

After twelve passages of the 1947 strain the macroscopic changes in inoculated membranes became more readily identifiable. Minute pocks 0·5–2·0 mm. in diameter were regularly observed, particularly when the membranes were examined 3 days after inoculation (see Pl. 8, fig. 3). Unless the inoculum was suitably diluted these lesions tended to coalese so that only a confluent generalized thickening was observed. Because of the minute size of the pocks, however, it was not possible to titrate the virus by this method. Histologically the lesions showed the same characteristic changes as those of the earlier passages.

Inoculation of yolk sac and amniotic cavity was not investigated, but after three passages in the allantoic cavity characteristic microscopical appearances were produced when the fluid was passaged back to the c.a. membrane.

Because young ducklings could be infected, attempts were also made to grow the virus in fertile duck eggs, but after one passage on the chorioallantoic membrane no histological or other evidence of infection was found.

The 1948 (blister fluid) strain was maintained for twenty-six passages on the c.a. membrane, and its behaviour was similar to the 1947 (blood) strain in every respect except that the macroscopic lesions were not so readily observed even after the egg adaptation. The 1949 (blister fluid) strain was passaged three times and the shearwater mite strain six times. All these strains regularly produced characteristic histological changes in the c.a. membranes, but recognizable macroscopic lesions were rarely found. Attempts to isolate the virus from rabbit mites and various other possible sources of infection on Skomer were unsuccessful even after repeated passages.

INOCULATION OF BIRDS

Shearwaters. In 1948 four normal juvenile shearwaters, taken from Skomer before the natural disease had appeared, were inoculated with a suspension of chorio-allantoic membrane inoculated with the 21st passage of the 1947 strain, two by scarification and two by intradermal injection of the webs. No blisters developed, but one bird died on the 9th day and two developed conjunctivitis and died on the 10th day after inoculation. The remaining bird showed no illness at any stage. Post-mortem examination of the dead birds revealed no abnormality, but virus was re-isolated from the spleen of one of the birds by inoculation of the c.a. membrane of fertile eggs. In 1950 a pair of juvenile birds were inoculated with reconstituted freeze-dried blister fluid collected in 1949. These birds remained well, and it was shown subsequently by inoculation of eggs that the dried material was inactive. After they had remained well for 1 month these two shearwaters were re-inoculated by scarification with a sterile filtrate of blister fluid from a moribund juvenile herring gull (*Larus argentatus*) collected on Fair Isle. After 4 days both shearwaters developed blisters on the webs which closely resembled those seen in the natural condition. Neither bird became seriously ill and the blisters healed completely.

Ducklings. Considerable difficulty was experienced in keeping ducklings in an infected animal house and the death-rate among controls was high in the early experiments, but later, as more experience of duckling management was obtained,

deaths among controls became much fewer. However, at all stages the test animals showed signs differing from those in the controls.

The material from the 1947 outbreak was passed to ducklings directly as an organ suspension from two shearwaters, received in the laboratory when sick from the disease, and as a suspension of C.A. membrane from the 9th egg passage. The animals were infected by scarification of 0·1 ml. of material into the dorsal surface of both compartments of the web of one foot. Typically, after 3 days, small blisters appeared along the scratch marks (see Pl. 8, fig. 4) often with oedema of the shank as well. In the early experiments all birds died and generally showed either consolidation of the lungs or at least gross congestion, usually in the mid-zone of one lung. (In the 1947 season similar lung consolidation was more commonly seen in post-mortems on shearwaters dying of the disease than in the later seasons.)

On direct passage the virus did not appear to adapt to the duckling and could not be recovered for more than three passages. An attempt was therefore made to adapt the virus by passing alternately in eggs and ducklings. After four pairs of passages the virus appeared to have greatly increased its ability to produce blisters in the ducklings, and in both the fourth and the fifth duckling passage, after intermediate egg passage, gross blisters were seen on the ducklings' webs exactly like those on the shearwaters'. However, on return to direct duckling passage the virus was again rapidly lost. This experiment was not repeated because the season when ducklings were available came to an end. Ducklings which recovered after blistering showed necrosis of the web, and after the necrotic area had separated a hole was commonly left in the web.

Pigeons. Twelve pigeons were inoculated by scarification into the plucked breast of 0·1 ml. suspensions of either egg or duckling passage material, and of these five developed definite superficial blistering in the inoculated area. These birds were rather listless, and, compared with controls which were inoculated with broth, they showed marked loss of breast muscle and very delayed feather regeneration. Blister fluid from pigeons produced typical inclusions when inoculated on to the C.A. membrane of fertile hens' eggs and convalescent serum from a pigeon which developed blisters after inoculation with 1947 duckling material gave a specific complement fixation to a low titre with an antigen prepared from chorio-allantoic membrane (see complement-fixation tests).

Fowls. No evidence of infection was found either in 8-week-old chicks after scarification into the plucked breast, or in two cockerels after scarification of material into the comb.

INOCULATION OF MAMMALS

Mice were inoculated with blood, blister fluid filtered through a 'Gradocol' membrane of 750 mμ A.P.D. and with a 10 % suspension of pooled liver, kidney and lung of a shearwater which was killed when moribund from the disease. Each material was inoculated into groups of four mice by the intraperitoneal, intracerebral and intranasal routes. Blind passages from two mice of each group by the appropriate routes at 4-day intervals, failed to cause any obvious ill health in the mice at the third passage. The remaining two mice inoculated intracerebrally with blister fluid

and two inoculated intracerebrally with organ extract died 17 days after inoculation, and brains from these mice were sterile on aerobic culture. Passage of suspensions of these brains diluted 10^{-2}, 10^{-3} and 10^{-4} by intracerebral inoculation to further mice failed to reproduce this effect. Attempts were also made to infect mice with egg-passage virus by intradermal inoculation into the pads, by scarification of the pads, and by intradermal inoculation into the tongue. No evidence of infection was obtained.

Guinea-pigs and rabbits. Attempts were made to infect recently weaned guinea-pigs and rabbits by intradermal inoculation of egg-passage virus into the pad, by scarification of the pad, and by intradermal inoculation into the tongue. One rabbit developed a small ulcer on the tongue at the site of inoculation after 10 days, and this healed completely in 5 days. This result could not be repeated, and no animals showed any pyrexia following inoculation. (Since wild rabbits living in close contact with infected shearwaters are apparently unaffected, the results with the rabbits were only to be expected.)

A cow. Pooled blister fluid from sick shearwaters was sent to the Foot and Mouth Disease Research Centre, Pirbright, where Dr Brooksby kindly inoculated it intradermally into the tongue of a cow but no vesicle or other abnormality developed.

FILTRATION EXPERIMENTS

Attempts to estimate the approximate size of the infective particle were made by filtration through 'Gradocol' membranes of varying pore sizes. 5 % suspensions of C.A. membranes infected with the 1947 strain were filtered and tested by inoculation of fresh C.A. membranes and by scarification of duckling webs. The development of ectodermal proliferation with typical inclusion bodies was taken to indicate infection in the inoculated membranes, but two blind passages were carried out when membranes were apparently uninfected. The appearance of vesicles at the site of inoculation was taken as a criterion of infection in the ducklings.

The results of these experiments are shown in Table 1, and it will be seen that the virus was much smaller than was anticipated and that the pore sizes used in the earlier experiments were too large. With chorio-allantoic inoculation the egg-adapted virus passed membranes with an A.P.D. of 48 mμ on two out of three occasions but failed to pass membranes whose A.P.D. was 31 mμ or less. These limits, after correction, suggest that the virus is between 20 and 30 mμ in diameter. Ability to pass 48 mμ A.P.D. filters was also shown by inoculation of ducklings, but membranes of smaller pore size were not used. A suspension of duckling web infected with the 1947 strain was also found to cause blisters in further ducklings after filtration through 48 mμ. The 1948 strains from blister fluid and mites also passed filters of this pore size.

NEUTRALIZATION TESTS

Chorio-allantoic membrane suspensions of the 20th egg passage of the 1947 strain were titrated in ducklings' webs and produced typical vesicles at a dilution of 10^{-7} but not 10^{-9} (Table 2). A neutralization test was then carried out using this

virus suspension diluted 10^{-6} and 10^{-7} with serum obtained from a shearwater in the acute phase of the disease, and a convalescent pool of serum from two birds which had survived the disease. Serum virus mixtures were incubated for 2 hr. at room temperature and then inoculated by scarification of duckling webs. The results (Table 2) show that the acute phase serum failed to prevent infection by virus suspension diluted 10^{-6} or 10^{-7}, while convalescent serum completely neutralized the actions of the virus at both dilutions.

Table 1. *Filtration of shearwater virus through Gradocol membranes of varying pore diameter with subsequent infectivity tests on* C.A. *membranes of chick embryos and duckling webs*

+ denotes specific histological changes in C.A. membranes. − denotes no specific histological changes in C.A. membranes after three passages. In duckling web experiments denominator indicates number of ducklings inoculated. Numerator gives number with vesicles. Figures in brackets indicate ducklings dying in the first 3 days before development of vesicles.

Source of virus ...	C.A. membrane						Duckling web
Tested in ...	C.A. membrane				Duckling web		Duckling web
Exp. no. ...	1	2	3	4	1	2	1
Unfiltered	+	+	+	+	(2) 2/4	4/4	4/4
Filtered:							
A.P.D. 400 mμ	+	.	.	.	.	.	.
A.P.D. 200 mμ	+	+	.	.	.	.	.
A.P.D. 130 mμ	.	+	.	.	(2) 1/4	.	.
A.P.D. 48 mμ	.	+	+	−	3/4	4/4	4/4
A.P.D. 31 mμ	.	.	−	−	.	.	.
A.P.D. 8 mμ	.	.	−	−	.	.	.
Control inoculated with broth	.	.	.	.	0/3	0/4	0/3

Table 2. *Titration of infected* C.A. *membranes by inoculation of ducklings and neutralization test with acute and convalescent shearwater serum*

Denominator indicates number of ducklings inoculated. Numerator gives number with vesicles. Figures in brackets indicate ducklings dying in first 3 days before development of vesicles.

Dilution of infected c.a. membrane suspension ...	$10^{-1 \cdot 3}$	10^{-5}	10^{-6}	10^{-7}	10^{-9}
Straight titration ...	4/4	(1) 3/4	.	(1) 2/3	0/4
Neutralization with:					
Acute serum	.	.	3/5	1/5	.
Convalescent serum	.	.	0/5	0/5	.

COMPLEMENT FIXATION TESTS

The supernatants from lightly centrifuged saline extracts of infected chorio-allantoic membrane, and of lung and liver from infected ducklings were used as antigens for complement-fixation tests. Various batches of antigen were made chiefly from the 1947 strain at different stages of egg adaptation. The acute phase shearwater serum and convalescent shearwater serum pool (as used in the

neutralization test) were used, and these were inactivated at 56° C. for 30 min. before titration with 2 M.H.D. of guinea-pig complement and the various antigens. Fixation was for 1 hr. at 37° C. before addition of sensitized cells. The results of tests done with these sera on separate occasions are shown in Table 3. 5 % duckling organ antigen and 5 % C.A. membrane antigen from egg passages, 8 and 20, all fixed complement with the convalescent serum but not with acute serum or

Table 3. *Complement-fixation tests with shearwater serum and antigens prepared from duckling tissues and* C.A. *membranes of fertile hens' eggs*

4 denotes complete fixation of 2 M.H.D. of complement, 3, 2 and 1 denote intermediate degrees of fixation and 0 indicates no fixation of 2 M.H.D. of complement.

Antigen	Acute stage serum		Normal serum		Convalescent serum				Antigen control
	5	10	5	10	5	10	20	40	
Duckling lung and liver 5 %, freshly isolated	2	0	.	.	4	4	2	0	0
Duckling lung and liver 5 %, from egg passage 10	1	0	.	.	4	4	2	0	0
C.A. membrane 5 %, passage 8	1	0	.	.	4	4	2	0	0
C.A. membrane 5 %, passage 20	.	.	0	0	4	3	1	0	0
Normal C.A. membrane 5 %	.	.	0	0	1	0	0	0	0
Serum control	1	0	0	0	1	0	0	0	.

normal serum. Passage 20 egg antigen also fixed complement with serum diluted 1:5 from a pigeon which had developed blisters after inoculation with virus. Antigens, made from the chorio-allantoic membranes of the 28th egg passage of the 1947 strain, failed to fix complement with the convalescent shearwater or any other serum tested.

HAEMAGGLUTINATION

20 % saline extracts of chorio-allantoic membranes inoculated with egg-adapted virus were tested for their ability to agglutinate human group O red cells, including cells treated with incomplete *Rh* antibody, and different batches of fowl cells (both susceptible and unsusceptible to haemagglutination by vaccinia virus). A poorly developed lipoid type of agglutination was sometimes seen at low dilutions of virus, but this was inhibited by normal human serum or rabbit serum diluted 1:100. Suspensions of infected duckling organs, both untreated and heated to 56° C. for 30 min., failed to agglutinate duckling cells or chicken cells.

SUMMARY AND CONCLUSIONS

Evidence for the isolation of a virus from this epizootic disease of shearwaters is based upon the reproduction of a similar condition in ducklings and pigeons and upon the development of characteristic histological changes with cytoplasmic eosinophilic inclusions in inoculated chorio-allantoic membranes of chick embryos. It was not possible to reproduce the original disease exactly by inoculation of shearwaters with egg-passage virus from the previous season because the birds did not develop blisters at the site of inoculation of the web. Nevertheless, three

out of four birds died and the agent was re-isolated from the spleen of one of them. The ability of the virus to cause blisters on duckling webs was neutralized by shearwater serum taken at the convalescent but not at the acute stage of the disease, and complement fixation was obtained with convalescent shearwater serum.

Previous studies on natural virus infections of birds have been mainly limited to psittacosis and ornithosis, the pox viruses, Newcastle disease virus and some of the neurotropic viruses. However, the failure to infect mice by any route, and the ability of the shearwater virus to pass through filters with an A.P.D. of 48 mμ, seemed to rule out the psittacosis group as a cause of the infection. The ability of the virus to cause vesicles and the intracytoplasmic eosinophilic inclusion bodies suggested a member of the pox group of viruses, but the small size of the agent renders a relation to either the pox group or Newcastle disease virus unlikely. Several of the neurotropic viruses which infect birds, for example, Japanese B virus, are about the size of the shearwater virus, but they do not cause the formation of inclusion bodies and they are all pathogenic for mice. Foot and mouth disease virus is also of small size and produces vesicular lesions, but it is unlikely that the shearwater virus is the same since it was unable to infect a cow or the pad of a guinea-pig, and it grew in eggs more readily than foot and mouth disease virus.

There has obviously been insufficient work on the agent to postulate a new virus, but the properties described do not justify its classification in any of the known virus groups. The name puffinosis which was originally suggested still seems suitable for this disease of *Puffinus puffinus puffinus*.

Our thanks are due to Dr D. Surrey Dane for helpful advice and co-operation and for permission to publish Pl. 8. We are also grateful to Mr R. Codd, late of Skomer Island, and Mr P. Conder of Skokholm Bird Observatory, and to Dr. J. Brooksby, of the Foot and Mouth Disease Research Centre, Pirbright.

REFERENCES

Miles, J. A. R. & Stoker, M. G. P. (1948). Puffinosis, a virus epizootic of the Manx Shearwater (*Puffinus p. puffinus*). *Nature, Lond.*, **161**, 1016.

Surrey Dane, D. (1948). A disease of Manx shearwaters (*Puffinus puffinus*). *J. Anim. Ecol.* **17**, 158.

Surrey Dane, D., Miles, J. A. R. & Stoker, M. G. P. (1953). Further observations on the epizootic of the Manx shearwater (*Puffinus p. puffinus*) on Skomer Island. *J. Anim. Ecol.* (In the Press.)

Van Rooyen, C. E. & Rhodes, A. J. (1948). *Virus Diseases of Man*, p. 92. New York: Nelson.

EXPLANATION OF PLATE

PLATE 8

Fig. 1. Blistered web of naturally infected juvenile shearwater.

Fig. 2. Section of ectoderm of infected chorio-allantoic membrane of fertile hen egg, showing hyperplasia and several cytoplasmic inclusion bodies. The arrow indicates a cell with numerous inclusions clustered around the nucleus. (Haematoxylin and eosin × 1000.)

Fig. 3. Macroscopic appearance of an infected chorio-allantoic membrane of fertile hen egg.

Fig. 4. Lesions on duckling's web.

(*MS received for publication* 3. VII. 52.)