AN INTESTINAL THREADWORM AS A RESERVOIR AND INTERMEDIATE HOST FOR SWINE INFLUENZA VIRUS*

A Confirmation and Amplification of Shope's Syndrome

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The purpose of the present research has been to explore further the virus-nematode relationships noted by the late Dr. Richard E. Shope. Our initial aim was to establish a practical laboratory model which would make possible an extension of his work under controlled conditions. The model system used in these studies involved the interactions of *Stronglyoides ratti* and swine influenza virus in the rat and mouse.

The role of various helminths in the transmission of viral and rickettsial disease, both as latent and active infections of the animal and plant kingdoms, has been well documented (1–11). The most complete study of this type involves the interaction of swine lungworms and the swine influenza virus (12–19).

In the 1930's, Shope carried out experiments on various vaccines and bacterins which might be used to protect swine against swine influenza. He demonstrated that

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bacterins of *Hemophilus influenzae* afforded a partial protection while a vaccine of swine influenza virus resulted in complete immunity (12–16). Subsequent investigation of the method of exposure of swine to the virus failed to elucidate how exposure occurred, and it was postulated that the swine were exposed to the virus in some unusual manner.

Further studies by Shope revealed that swine lungworms (either *Metastrongylus elongatus* or *M. pudendotectus*) acted as carriers of the swine influenza virus though they went through several intermediate stages outside swine (17). These experimental findings were borne out subsequently in studies of natural infections and studies on the effect of stress and/or "cold spells" on the frequency of outbreaks (18, 19). Parallel studies with hog cholera virus have shown it to be also transmitted by the swine lungworm in a manner similar to the swine influenza virus (20).

Experimentation and observations over the last 30 yr have substantiated Shope's concept of the epidemiology of swine influenza, in which the virus is maintained and spread in a "masked form" by a lungworm intermediate host. To further bear out Shope's findings, Sen et al. repeated his work using pathogen-free, antibody-free pigs (21). The results of this work were essentially the same as those of Shope. Pathogen-free, colostrum-deprived pigs appeared to be more susceptible hosts. Provocation was effected during all seasons of the year. Ascaris extract or the migration of *Ascaris suum* furnished the needed provocation.

Materials and Methods

Virus.—The strain of influenza virus, used in these studies, A/swine/1976/31 M_{38} E_{48} M_6, was prepared on 26 July 1965 by the Respirovirus Unit, National Communicable Disease Center, and obtained from them in September 1965. This strain represents a subculture of an isolate made by Shope in his early work. The letters M and E and the numerical subscripts indicate the order and number of mouse and egg passages, respectively.

The virus was passed four additional times in mice prior to the preparation of a "stock" in mice for use in these studies.

Passage and Storage of Virus.—The swine influenza virus was propagated in mice by anesthetizing the animal lightly with ether and administering 0.05 ml of a 10% lung supernatant intranasally using a 0.25 ml syringe equipped with a 25 gauge needle. After death, the lungs were removed and a 10% suspension prepared by grinding the lungs with mortar and pestle with the addition of sterile sand. The suspensions were then centrifuged at 138 *g* for 10 min using either saline or tryptose phosphate broth as a diluent. The centrifuged supernatants were stored at −89°C pending use.

Titration of Infectious Virus.—Infected mouse lung supernatants were serially diluted in 10-fold steps in chilled tryptose phosphate broth. Mice, 21–28 days of age, were inoculated intranasally with 0.05 ml of the mouse lung supernatants while under ether anesthesia. Usually 5 mice, but on occasion as many as 20, were used per dilution. Rats were also inoculated in the above manner.

Lungs from mice dying between the 3rd and 12th days postinoculation were examined by hemagglutination (HA) as an indication of the presence of hemagglutinins. A pool of lung suspensions from each titration were examined by hemagglutination inhibition (HI) to determine the specificity of the observed HA.

The fifty per cent end point was calculated by the method of Reed and Muench (22).

Mice and Rats. The mice and rat used in this study were specific pathogen-free stock.

These animals were caesarean-originated and barrier-sustained (COBS) until shipment.[1] Mice were received at 21 days of age; the rats were weanlings approximately 4 wk of age. Upon receipt, the mice and rats were placed in sterile cages on sterile pine shavings and fed commercial laboratory chow.

Helminth. Prior to receipt, the *S. ratti* isolate used in these studies had been passaged 65 times in domestic rats.[2] A diagramatic sketch of the life cycle of the helminth is shown in Fig. 1.

Rats infected with *S. ratti* were maintained in wire bottom cages with removable collecting trays. To prevent drying of the fecal pellet, trays were lined with paper towels moistened so that they did not dry between fecal collections. Collections of fecal material were made in the

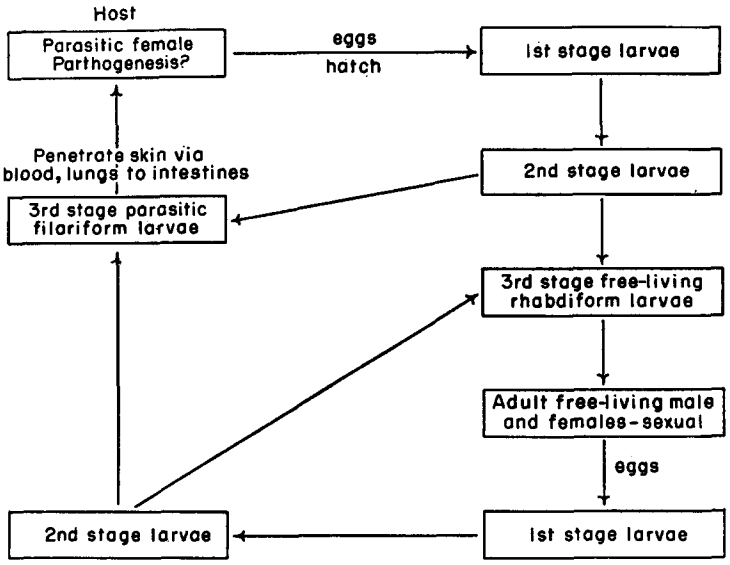

FIG. 1. Life cycle of *Strongyloides ratti*, Sandground, 1925.

morning; however, a somewhat better yield of *S. ratti* was obtained if an additional collection was made in the late afternoon.

Fecal pellets were placed in a culture dish and mixed (not crushed) with an equal amount of granular charcoal; very coarse granular and powdered charcoal were found to be unsuitable. This mixture was dampened with sufficient water to saturate the charcoal, but not to the degree that water ran off the mixture. It was covered to prevent evaporation and maintained at 27°C for 3 or 4 days.

Larvae were separated from the culture media using the Baermann apparatus described by

[1] Charles River Breeding Laboratories, Inc., North Wilmington, Mass. (Names of commercial manufacturers and other trade names are provided for identification only, and their inclusion does not imply endorsement by the Public Health Service or the U. S. Department of Health, Education, and Welfare.)

[2] Dr. Frank F. Katz, Seton Hall University, South Orange, N. J.

Cort et al. (23). The fecal culture was allowed to stand in this apparatus for 1 hr at which time approximately 15 ml of tap water was drawn off from the bottom of the funnel. The larvae became immobile and nonviable if allowed to remain in the collecting tube for longer than 1 or 2 hr. Samples of water were removed at hourly intervals until larvae were no longer evident. The 15–20 ml samples containing larvae were transferred to 15 × 100 mm Petri dishes and held at 27°C until needed. Larvae were found to remain viable, as judged by motility, for 2–3 wk under these conditions and only motile larvae were used for subsequent studies.

Subcutaneous Inoculation.—Amounts of larvae and/or virus suspensions varying from 0.2 ml to 0.3 ml were inoculated subcutaneously into mice and rats in either the inguinal area or the loose skin of the dorsal neck region.

Virus Isolation Techniques.—

"Egg-bit" technique: This technique was first described by Fulton and Armitage (24) and later adapted by Fazekas de St. Groth et al. for the titration of virus and assay of neutralizing

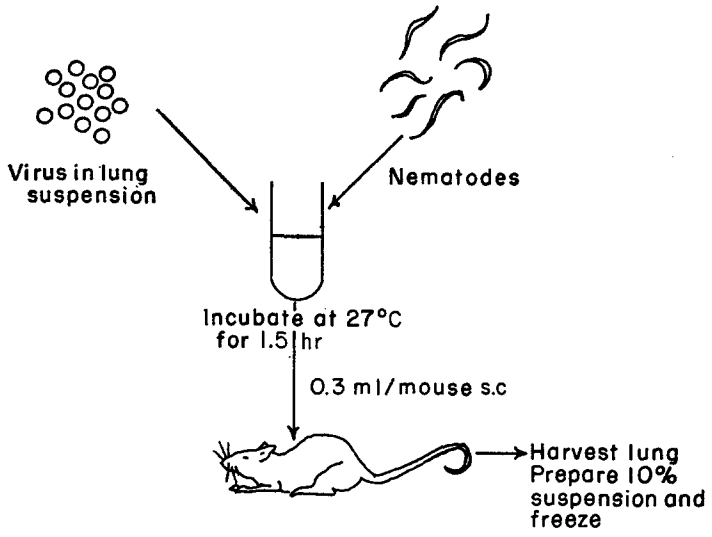

FIG. 2. Experiment I. Mixing of virus and helminth in vitro and inoculation into COBS mice.

antibodies (25–29). Briefly, the technique involved the cell culture of chorioallantoic membrane on a small piece of egg shell in a depression plate with a maintenance medium. Material suspected of containing virus was inoculated in this system. After incubation, the shells were removed and the medium tested for hemagglutinins. Any HA-positive (frank reaction or "buttons" which did not slip when the plate was elevated 60–70 degrees from base) samples observed were collected and stored at −89°C for further study.

15 units of mycostatin/ml of growth medium was used during the latter portion of the study to retard mycotic contamination without apparent detriment to the system.

Egg inoculations: Embryos, 9–11 days old, were each inoculated via the allantoic route with 0.2 ml of material suspected of containing virus. At least three eggs per sample were inoculated. The eggs were incubated at 37°C for 72 hr and allantoic fluid was then harvested and stored at −89°C.

Hemagglutination (HA) and Hemagglutination Inhibition (HI): The Microtiter[3] technique

[3] Cooke Engineering Co., Alexandria, Va.

was used to determine the presence of hemagglutinins (30). The alpha HI technique (constant serum-varying virus) designed for viral identification was used throughout this study. The Microtiter technique was also used for this test. A dilution of A/swine/1976/31 hyperimmune serum was prepared at a concentration which yielded the most specific HI reaction for use in these studies. The end point was read as the highest dilution of virus not inhibited by the dilution of antiserum used.

TABLE I

Virus Recovery From Mice Inoculated Subcutaneously With Nematodes After Viral Exposure In Vitro

Trial	Mice per group	No. of mice yielding virus per inoculation		
		60 virus-exposed larvae	60 unexposed larvae	80 LD_{50} virus only
1	4	0	0	0
2	5	0*	0	5‡
3	4	0	0	0
4	4	0	0	2§
5	4	2‖	0	0
6	10	0	0	0
7	5	0	0	0
8	5	0	0	0
9	10	0	0	0
10	18	1	0	0
Totals	69	3	0	7
Percentage		4	0	10

* All died in 24 hr. Death was probably due to lack of larval cleanliness since no hemagglutinins were demonstrated.

‡ All showed HA in lung suspension; no HI performed.

§ Two of four showed HA of lung suspension and were confirmed by HI as swine influenza virus.

‖ Three of four showed HA of lung suspension and two were confirmed by HI as swine influenza virus.

EXPERIMENTAL DESIGN AND RESULTS

Experiment I. Mixing of Virus and Helminth In Vitro and Inoculation into COBS Mice. (Fig. 2).—

S. ratti larvae were exposed in a 15 × 100 mm Petri dish to approximately 80 mouse intranasal LD_{50} of swine influenza virus for 1.5 hr at 27°C. A 0.3 ml aliquot containing 60 larvae was then inoculated subcutaneously into either the inguinal area or loose skin at the back of the neck. Appropriate controls consisting of larvae not exposed to virus and of virus alone were inoculated into mice as described above. The mice were killed from 6–8 days after inoculation and lung suspensions examined for the presence of swine influenza virus.

In 3 of 69 (4%) mice inoculated with virus plus helminth, influenza virus could be recovered from lung tissue and identified as the virus strain inoculated (Table I). It was similarly shown that influenza virus of the strain inoculated could be recovered from lung tissue from 7 of 69 (10%) mice injected subcutaneously with virus alone.

TABLE II

Effect of Increased Subcutaneous Virus Dosage on the Frequency of Virus Recovery from Mouse Lungs

Virus* dilution	ID_{50} virus-inoculated	Mice per group	Lungs with virus recovery
10^{-1}	56,230	15	3
10^{-2}	5,623	15	0
10^{-4}	56	13	3

* Virus titer 5.75 logs.

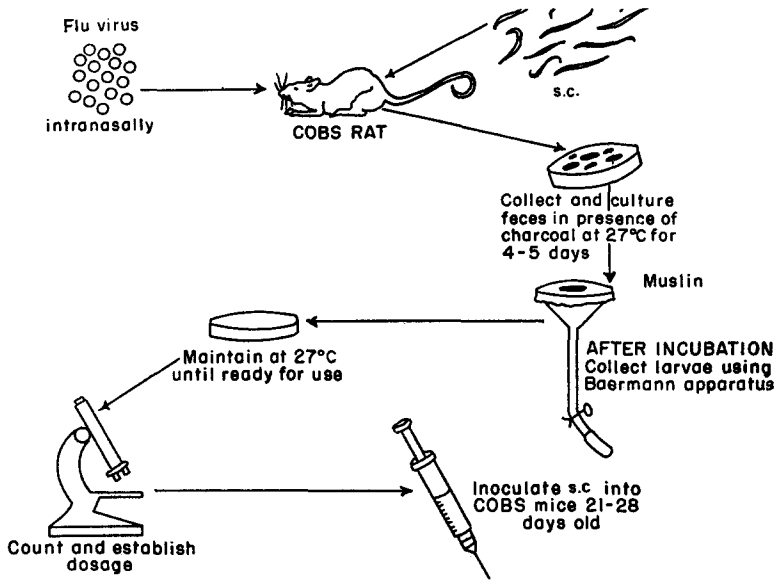

FIG. 3. Experiment II. Infection of mice with larvae exposed to swine influenza virus-infected rats.

At no time during these studies could swine influenza virus be recovered from the lungs of mice receiving larvae not exposed to virus in dosages comparable to other groups.

A group of mice were inoculated subcutaneously with varying dilutions (Table II) of the virus alone to determine if the amount of virus inoculated affected the frequency with which virus could be recovered from the lung. No correlation could be demonstrated between subcutaneous dosage and the frequency with which virus was recovered from the lungs.

From the data obtained, it could not be established whether the presence of virus in the lungs of mice inoculated subcutaneously with virus-exposed larvae represents a mechanical transmission of virus by the larvae or the presence of virus by other means.

Experiment II. Infection of Mice with Larvae Recovered from Swine Influenza Virus-infected Rats. (Fig. 3).—

A group of COBS rats were infected via the intranasal route with 0.2 ml each of a 10% suspension of mouse lungs containing 87,500 mouse LD_{50}. 72 hr later, these rats were given 1000 *S. ratti* larvae each via the subcutaneous route. The nematode offspring obtained from rats inoculated in the above manner will be referred to as "exposed." Similarly, offspring obtained from rats not infected with swine influenza virus will be termed "unexposed."

TABLE III

Virus Recovery from Mice Inoculated Subcutaneously with Larval Offspring of S. ratti Obtained From Influenza Virus-Infected Rats

Day of larval recovery*	Larval dose per mouse	Rate of virus recovery from mice inoculated with	
		Exposed larvae	Unexposed larvae
4–6	40–80	25/142‡	0/44
9	100–160	2/40	0/20
Totals		27/182	0/64
Percentage		15	0

* Time in days postinoculation that exposed worms were recovered from shedder rats.

‡ Number of virus recoveries per number of mice inoculated.

After establishment of a dual nematode-virus infection in the rats, the offspring of those worms exposed to virus in vivo were collected and used in a number of attempts to determine whether or not the worm harbored the virus and could pass it on to COBS mice. This was accomplished by inoculation of exposed *S. ratti* into mice via the subcutaneous route and examination of the lungs of these animals 5 days postinoculation for the presence of virus.

Single fecal pellets were collected from rats shedding virus-exposed and unexposed worms, placed in sterile tubes, and emulsified in tryptose phosphate broth. These pellets were centrifuged for 30 min at 1680 *g* in a refrigerated Servall centrifuge and the supernatant was collected and screened for the presence of virus by "egg-bit" technique. All results were negative.

The offspring of viral exposed worms successfully transmitted the virus to 27 of 182 (15%) mice while unexposed worms failed to transmit virus in an additional 64 mice (Table III).

Rats shedding exposed worms did not pass detectable quantities of influenza

virus in their feces. It would be logical from ths data to assume that the virus was being carried by *S. ratti*, either within the worm or adhering to the cuticle.

Experiment III. In Vitro Attempts to Demonstrate Swine Influenza Virus in Larvae Recovered from Influenza-Infected Rats.—

Approximately 2×10^4/ml of virus-unexposed and exposed larvae were treated with A/swine/1976/31 antiserum, washed thoroughly with saline, and triturated in a micro cup surrounded with ice using a Virtis "45" homogenizer set of maximum speed for 5 min. The samples were further divided and one aliquot of each was treated with specific antiserum. These samples were then cultured on "egg-bit" in an attempt to detect the presence of influenza virus.

An HA-positive agent was recovered, in one of two attempts, from the triturated exposed larvae to which antiserum had not been added. This agent was subsequently identified as swine influenza virus by HI.

DISCUSSION

Certainly the action of the complex here described on the mouse has not been elucidated beyond the ability of subsequent generations of *S. ratti* larvae to transmit an unknown amount of virus from one host to another. These results indicate that it is possible, using the described virus-nematode system, to study virus infections which follow the same basic pattern of transmission seen in swine influenza.

The duration of the influenza infection in the rat would appear to play an important role in the success of larvae infected with virus. Gildemeister noted that a subclinical infection of rats by swine influenza virus may occur and that the infection may persist for as long as 30 days (31).

The ability to demonstrate swine influenza virus in mice during Experiment I in the absence of worms has been difficult to explain since, classically, influenza virus has not been found to be infective via the subcutaneous route. It is not impossible for this observation to represent an intranasal infection as a result of mice "sniffing" inoculation sites where an infective dose of virus might have remained on the skin. Similarly, the disruption of the capillaries at the inoculation site would conceivably be sufficient to allow virus to migrate and localize in the lung.

It should be stressed again that none of the COBS mice showed areas of lung consolidation nor any sign of frank disease in either Experiments I or II, though virus was recovered with regularity in both experiments.

An apparent inverse ratio was observed between the number of exposed larvae inoculated and the rate of virus transmission (Table III). Examination of the data revealed, however, that the effect of larval dose on the rate of virus transmission was not significant. However, if one compares the day of larval recovery with the rate of virus transmission, the observed difference is signifi-

cant. Exposed larvae collected 4–6 days postinoculation transmitted virus to 17.6% (25 of 142) of inoculated mice. In spite of increased dosage, larvae collected 9 days postinoculation transmitted virus to only 5% (2 of 40) of inoculated mice.

Two alternative explanations may be offered in an attempt to account for the observed difference. It may be hypothesized that infection of the adult worm results in biological transmission of virus to the larval progeny. This cycle would be self-limiting and result in a progressive decline in the yield of exposed viable larvae if viral infection of the adult nematode and/or the larvae terminates fatally. This hypothesis agrees with the findings of Westcott wherein he reported that the presence of influenza virus reduced the numbers of viable *Nippostrongylus brasiliensis* larvae in axenic culture, and speculated that virus infection resulted in death of the nematode.[4] Furthermore, this hypothesis is consistent with our observations that unexposed larvae were obtained from shedder rats for as long as 30 days, while it was difficult to obtain exposed viable larvae for periods in excess of 14 days.

As an alternative to the above, the hypothesis may be advanced that a mechanical transfer of virus occurs in which infected adults are able to shed virus-infected larvae for only a limited period of time. Transfer of virus in this manner would be limited by the amount of virus initially associated with the adult nematodes.

Although the data presented do not allow one to make a choice in favor of either hypothesis, some form of biological transmission seems most probable. The strongest evidence in support of biological transmission is the presence of detectable amounts of virus in the postexposure generation of the parasite.

It was possible to recover swine influenza virus in 15% of the COBS mice inoculated with virus-exposed larvae under controlled laboratory conditions. These data compare favorably with those obtained by Shope (17). To the authors' knowledge, the isolation of swine influenza virus from exposed worms represents the first recovery of this virus from nematodes which were exposed to virus experimentally in vivo.

SUMMARY

A laboratory model exemplifying Shope's concept of virus carriage by helminths was developed using *Strongyloides ratti* (Sandground, 1925), swine influenza virus, and caesarean-originated, barrier-sustained (COBS) mice and rats.

It was shown that *S. ratti* can act as a carrier of swine influenza virus and infect mice, despite the fact that the nematode has undergone a complete life cycle after exposure to virus in infected rats.

[4] Westcott, R. B. 1966. Personal communication.

COBS rats were inoculated with the virus via the intranasal route and subsequently infected with *S. ratti.* The larvae and eggs found in the feces collected from these rats were allowed to develop in vitro. These second generation filariform larvae were then inoculated subcutaneously into COBS mice. At necropsy, 5–8 days postinoculation, swine influenza virus was isolated from 15% of the mouse lungs. In control studies, swine influenza virus was not isolated from the feces of the COBS rats which served as a source of both unexposed and exposed *S. ratti.* Swine influenza virus was recovered in vitro from *S. ratti* homogenates. It is suggested that this laboratory model be used to study more closely the various aspects of the ecology of virus-helminth relationships in vertebrate hosts.

The authors wish to acknowledge the advice and suggestions of the late Dr. Richard E. Shope during the initial planning of this venture. His comments were directed toward simplicity of approach and were very revealing in our exploration of this complex interrelationship.

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