A STUDY IN VITRO OF COMPONENTS IN THE TRANSMISSION CYCLE OF SWINE INFLUENZA VIRUS*, ‡

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In the course of investigation on swine influenza, Shope (1) and Lewis and Shope (2) isolated swine influenza virus and *Hemophilus influenzae suis* from the lungs and bronchial secretions of cases. Shope (3) subsequently showed that the primary etiologic agent was the virus.

However, certain characteristics of epizootics of influenza in swine were difficult to explain on the basis of transmission of virus and bacterium from swine to swine by contagion (4). For example, epizootics of swine influenza have been observed yearly in the Midwestern United States since 1918, but only in the late fall and early winter months. During that period of sudden changes in climate to wet, cold weather, outbreaks may occur that involve simultaneously herds so widely separated from each other that the likelihood of transmission of the disease by contact seems remote. Moreover, within a herd, a high proportion of the susceptibles may become ill on the same day.

From such observations and from data obtained in a series of intricate experiments (5–8), Shope advanced the hypothesis that swine influenza virus persists in an intermediate host and reservoir, the swine lungworm, which ultimately transmits virus to swine after completing part of its life cycle in earthworms. During passage in lungworms and earthworms, virus is said to exist in a "masked" state, since it cannot be demonstrated directly. Virus may be "unmasked" so that it will produce disease and become capable of isolation and identification, by application of suitable provocative stresses to animals prepared by ingesting the larval stages of infected lungworms (6). One such stress is exposure to inclement weather (9). Shope concluded that the simultaneous appearance of swine influenza in herds widely separated from each other could best be explained by the "unmasking" of virus with the onset of cold weather.

Attempts to confirm Shope's observations or to test his hypothesis by further studies have been limited (10) since such investigations are hampered by the

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great cost of facilities suitable for maintaining large numbers of swine in strict isolation. In an effort to simplify experimental requirements the present investigation was initiated. The goal was to develop and use *in vitro* techniques for studying the reactions of swine influenza virus with components of the virus transmission cycle proposed by Shope.

This report summarizes new information about swine influenza virus in which: (*a*) the presence and characteristics of inhibitors of viral hemagglutination were studied using extracts prepared from lung, serum, or pulmonary mucus of swine, from swine lungworms, and from earthworms; (*b*) the fate of infectious virus was followed in cultures prepared from swine lung, swine lungworms, or earthworms. "Masking" or "unmasking" of virus was not demonstrated in these experiments.

Materials and Methods

Virus.—The strains of influenza A virus employed were: Asian (AA/23/57-E[1] 12), A-prime (AA/1/57-E4), A(PR8/34-F8M593E170), Swine 1976 ('35-M38E33), Swine November ('53-M1E12), and of influenza B virus, Lee ('40-F8M137E170). The strains of human origin were from the files of the Strain Study Center, Commission on Influenza, Virus Laboratory, School of Public Health, Ann Arbor, Michigan. The swine strains were obtained from Dr. R. E. Shope, The Rockefeller Institute, New York City.

Titrations of Egg Infectivity.—The procedure followed has been previously described (11). 50 per cent infectivity titers (EID_{50}) were calculated by the method of Reed and Muench (12). Titers are expressed as the reciprocal of the final dilutions employed.

Hemagglutination and Hemagglutination-Inhibition Titrations.—Measurements were carried out by a standard procedure (13).

Indicator Virus.—The Lee strain was enzymatically inactivated by heating at 56°C for 30 minutes. Swine 1976 strain was treated with versene (tetrasodium salt of ethylenediaminetetraacetic acid) in a final dilution of 1:80 with four hemagglutinating units of virus. Controls for such indicator virus preparations were provided by measurement of the levels of inhibition of hemagglutination obtained with serial twofold dilutions of a 10 per cent suspension of crude ovalbumin in saline.

Destruction of Inhibitors of Hemagglutination in Sera and Tissue Extracts.—Inhibitors of hemagglutination were inactivated by the addition of two volumes of M/90 potassium periodate and incubation at 4°C for 18 hours. Excess periodate was then inactivated by two volumes of 10 per cent glycerol in saline.

Swine Lungs, Pulmonary Mucus, Sera, and Swine Lungworms.—Lungs were collected from swine slaughtered at a local abattoir. Bronchi of the diaphragmatic lobes were dissected, and mucus was obtained by scraping their walls. Swine lungworms were removed from bronchi with forceps. Extracts of swine lung, lungworms, or mucus, were prepared in 0.15 M saline at 4°C, and were clarified prior to use by centrifugation at 1500 RPM using a model 2 International centrifuge. Blood was obtained aseptically, the serum separated, and stored at −20°C. For tissue culture, only antibody-free serum, as tested by hemagglutination-inhibition, was employed.

Maintenance of Adult Lungworms In Vitro.—Adult female lungworms were washed three times in large amounts of Hanks' balanced salt solution (14) containing 100 units each per

[1] The letters E, F, and M, and their numerical subscripts describe the passage history of each strain in eggs, ferrets, and mice.

ml of penicillin, streptomycin, and mycostatin. They were placed in Erlenmeyer flasks containing 2.7 ml of medium composed of balanced salt solution, lactalbumin hydrolysate (Nutritional Biochemicals Corporation), and horse serum (Difco TC, Difco Laboratories) in the proportion per 100 ml of 79.5, 0.5, and 20 parts, respectively. Flasks were incubated at 37°C. Under these conditions, lungworms could be maintained for at least 3 months if the medium was changed at 5 day intervals.

Preparations of Epithelial Cell Cultures from Lung.—Cultures of epithelial-like cells were prepared from swine lung according to a modification of the method of Youngner (15). 5 to 10 mm^3 sections of the diaphragmatic lobes were washed three times in balanced salt solution and added to a 250 ml flask containing approximately 50 ml of 0.25 per cent trypsin (Difco, 1:250) at 37°C. Tissue fragments were agitated for 10 minutes by a magnetic stirrer. The cell suspension obtained was decanted and fresh trypsin was added. The cycle was repeated six times. Fluids of the last five extractions with trypsin were collected and centrifuged at 600 RPM for 10 minutes. The supernatant was discarded, and the cells were resuspended to a final concentration of 2,000,000 cells per ml in a mixture of eight parts Eagle's basal medium (16), 1.5 parts swine serum, and 0.5 parts of chicken embryo extract (50 per cent). 5 ml of cell suspension were added to 3 ounce prescription bottles (sani-glas), which were incubated in a stationary position at 37°C. On the 3rd or 4th day, unattached cells were removed, and the medium replaced. At that time cells attached to the glass wall were usually small and discrete. However, sheets of contiguous epithelial-like cells were formed by the 6th to 10th day that could be maintained for 4 weeks, providing regular changes were made in the medium at 2 or 3 day intervals.

Earthworms.—Adult earthworms, obtained from a local farm and free from lungworm larvae, were placed in enamelware pans containing a mixture of moist soil and leaves, and were kept at 14°C or at 25°C. Saline extracts of earthworms were prepared by the procedure previously described. Cultures of the Maitland type (17) were made from minced tissues in medium of the same composition as that used for lungworm cultures.

EXPERIMENTAL

Investigations on the Presence and Properties of Inhibitors of Hemagglutination.—Heat-stable inhibitors of hemagglutination of influenza virus are regularly found in tissues that support the multiplication of virus (18–20). However, they also occur in a wide variety of insusceptible tissues and biologic fluids (21–23). Hence, the presence of such inhibitors clearly does not permit the conclusion that a tissue will support multiplication of viruses. On the other hand, failure to detect inhibitors in tissues suggests that they are insusceptible to infection by influenza virus.

To obtain evidence, then, on the question of whether lungworms or earthworms can support viral multiplication, experiments were carried out to ascertain whether extracts of these worms would inhibit hemagglutination. In addition, simultaneous measurements were made of the inhibitors in swine lung, mucus, and serum. Levels of inhibition were determined using active Lee and Swine 1976 strains as well as heated Lee, and versene-treated Swine 1976 preparations. One portion of each extract was tested as prepared; another, after heating in a boiling water bath for 2 minutes.

The data are presented in Table I. Lungworm extracts were found to contain

inhibitors detected only with indicator virus. The inhibitory activity was heat-stable. In contrast, earthworm extracts did not contain inhibitors detectable under the conditions employed. The demonstration that heat-stable inhibitors are present in extracts of swine lungworm but absent in extracts of earthworms indicates that the cells of lungworms may possess surface receptors for attachment of virus while those of earthworms do not. Such receptors could be important in the first stage of viral multiplication.

The findings with swine lung extract are in general like those previously described using "purified" inhibitor obtained from swine lungs (24). Levels of

TABLE I

Inhibitors of Hemagglutination Present in Extracts of Swine Lungworms, Earthworms, and Lungs, Lung Mucus, or Serum of Swine

Material	Treatment	Active Swine 1976	Versenated Swine 1976	Active Lee	Heated Lee
5 per cent swine lungworm extract	Untreated	<2*	512	<2	64
	Heated	<2	512	<2	128
10 per cent earthworm extract	Untreated	<2	<2	<2	<2
	Heated	<2	<2	<2	<2
10 per cent swine lung extract	Untreated	128	1024	16	256
	Heated	16	512	<2	64
10 per cent mucous extract	Untreated	16	512	8	64
	Heated	16	1024	8	128
Swine serum	Untreated	<10	40	<10	20
	Heated	<10	10240	<10	<10

* Hemagglutination-inhibition titers.

inhibition were consistently lower after boiling the lung extract. However, the inhibitors in pulmonary mucus appeared to be relatively heat-stable. Those in swine serum showed the remarkable characteristic of exhibiting, after boiling, lower levels when tested with heated Lee virus, but considerably higher levels when tested with versene-treated Swine 1976 strains.

The differences in the reactions after boiling of the inhibitors in swine serum and in extracts of swine lung serve to differentiate these inhibitors from each other and from those present in extracts of swine lungworms and pulmonary mucus. The biologic significance of these differences is unknown.

Further Characterization of Heat-Stable Inhibitors in Extracts of Lungworms, of Lung, and of Pulmonary Mucus.—The action of viral enzyme and potassium periodate upon these materials was tested to determine whether the inhibitors behaved like "Francis" inhibitors (21).

Extracts of lung, lungworms, and mucus were diluted to contain equal amounts of inhibitor reactive with versene-treated Swine 1976 strain to a titer of 1:128. Eight hemagglutinating units of active swine virus were added to an equal volume of each suspension. Saline was added to control preparations. At 10 minute intervals, samples were heated in a boiling water bath to destroy enzyme activity and hemagglutinin. The levels of residual inhibitor were then measured by an indicator virus.

The data in Fig. 1 are presented as the geometric mean values of three experiments. Upon treatment with eight units of active virus, the inhibitory activity

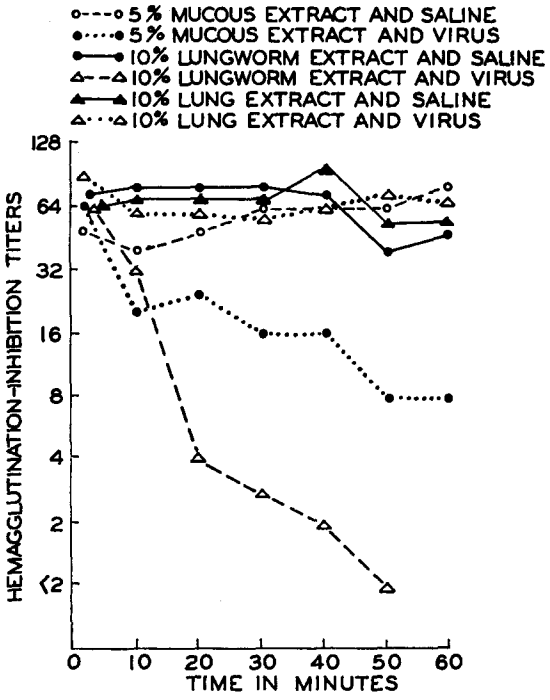

FIG. 1.

present in mucous and lungworm extracts declined progressively, while that in lung extract remained constant. However, in other experiments, inactivation of inhibitors in swine lung extracts could be demonstrated by using 320 hemagglutinating units of active virus in the test. The results indicate that inhibitors in extracts of lungworms are most susceptible to viral action, those in pulmonary mucus less so, and those in extracts of swine lung least susceptible. Complete destruction of the inhibitors of hemagglutination in each of these three extracts was obtained by treatment with potassium periodate.

The results indicate that inhibitors present in mucous, swine lung, and lungworm extracts have properties in common with receptor-like substances, *i.e.*,

higher levels of inhibition against heated virus, heat stability, and destruction by virus enzyme and potassium periodate. It may be concluded that presence of such receptor substances would favor the adsorption of virus to lungworms as it does to lung.

Fate of Infectious Virus Added to Lungworms In Vitro.—In preliminary experiments suitable conditions were found for maintaining adult lungworms in tissue culture media for prolonged periods of time. This observation made it possible to test whether lungworms can adsorb virus and whether evidence for multiplication of virus in lungworms could be established.

For these purposes, the following experiments were carried out.

One set of four flasks containing eight female lungworms in 2.7 ml of media per flask was inoculated with 0.3 ml of undiluted allantoic fluid, containing $10^{8.5}$ EID_{50} of swine strain, and placed on a shaker at 37°C. After 30 minutes and after 3, 6, and 10 days, one flask from each set was removed and the amount of virus present in the medium of these flasks was measured by titration in embryonate eggs and by hemagglutination. At the same intervals, lungworms were removed from the flask of the first set, washed four times in large volumes of cold buffered saline, ground in sufficient infusion broth to yield a 1 per cent suspension, and the virus content therein determined by egg infectivity.

The results of a typical experiment are shown in Fig. 2. In the flasks containing lungworms, the concentration of infectious virus in the tissue culture medium declined progressively at what appeared to be a uniform rate. In the control flasks which did not contain lungworms, the rate of decline of infectivity was faster. The slope of the curve of residual infectivity was steepest between the 3rd and 6th day. The infectivity of 1 per cent suspension of washed lungworms was initially low, and after the 3rd day, the rate of decline in infectivity seemed to accelerate, and became faster than that of the supernate. While the rate of decline of infectivity in suspensions of lungworms appeared to be intermediate between that of control flasks and of the supernate, at 10 days infectious virus could not be recovered from the worms. As measured by hemagglutination, there was no increase in the amount of virus present in the supernate, even when the period of observation was extended to 3 weeks. Taken together, these findings indicate that intact lungworms can adsorb small amounts of virus but they give no direct evidence that infectious virus can multiply in lungworms and be released into the suspending medium.

The observation that lungworms appear to slow down the rate of inactivation of infectious virus in the medium is an intriguing one. This effect could be enhanced by increasing the number of lungworms placed in each flask, and was abolished when the worms were removed, were killed by heat or by freezing, or when they were minced or ground. The mechanism of prolongation of survival of infectivity in the presence of lungworms is unknown. One possible explanation for that observation is that the level of infectivity in the supernates is supported by the release of virus which had multiplied in lungworms.

However, all attempts to demonstrate a net increase in infectious virus in suspensions of lungworms exposed to different concentrations of virus for intervals as short as 12 hours and as long as 3 weeks were unsuccessful. It might be considered that failure to demonstrate a net increase in infectious virus in lungworms does not exclude the possibility that virus multiplies in lungworms, but on the contrary would be the expected result since, theoretically, "masking" of virus in lungworms would render it non-infectious for eggs. However, it

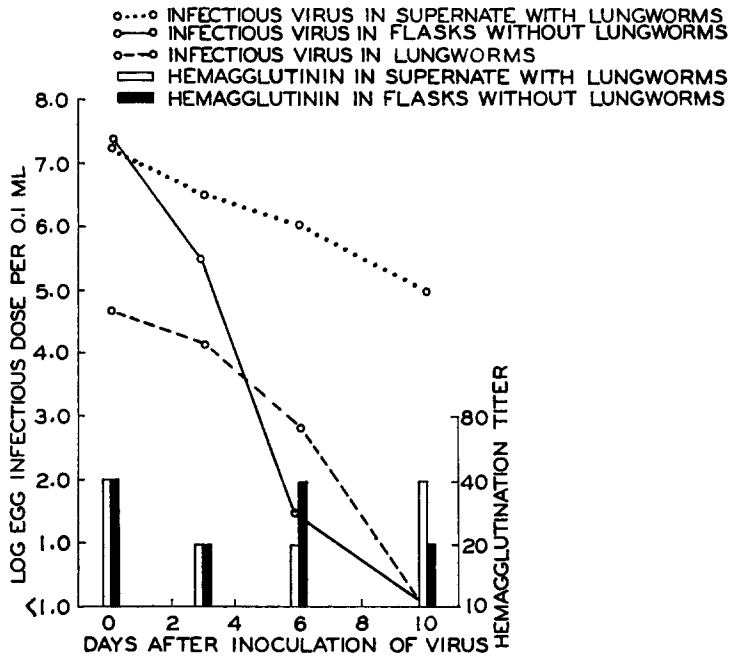

FIG. 2.

would be awkward to explain by that hypothesis why one portion of the virus newly synthesized in lungworms becomes masked, while another is released into the supernate in an infectious form. Moreover, as stated previously a net increase of virus in the supernate was not found by hemagglutination, although the reservation may be held that a small amount of newly synthesized virus was released into the supernate, but could not be detected by the methods employed.

An alternate explanation which seems more likely can be offered for the prolongation of survival of infectivity in the presence of lungworms. It has been repeatedly observed in this laboratory that virus which has eluted from cellular receptors is more resistant to inactivation by heat. These observations

suggest the possibility that under the conditions of the present experiment, the virus present in the supernates had been partially stabilized by adsorption to and elution from the surface receptors present in lungworms. Such receptors may even regenerate in living lungworms and thereby permit the occurrence of repeated cycles of attachment and release. Further work is needed to establish the final interpretation of the phenomena observed.

Fate of Infectious Virus Added to the Swine Lung In Vitro.—Another aspect of the present investigation was to ascertain the usefulness of cells obtained from swine lung as a substitute for the whole pig in investigations on the swine influenza cycle. As described in the section of Methods, it was found possible to obtain monolayer sheets of epithelial cells in prescriptio n bottle

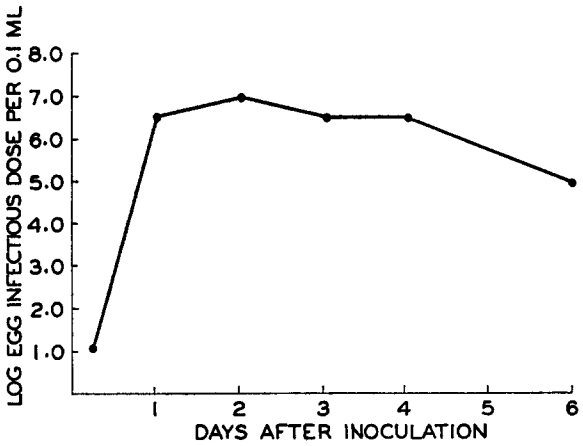

FIG. 3.

within 6 to 10 days after addition of a cell suspension. With these cells, growth of influenza viruses was demonstrated using the following procedures.

Cell monolayers were washed twice with balanced salt solution and inoculated with virus by adding 0.5 ml of virus dilution. 4.5 ml of balanced salt solution were added after the inoculum had spread over the surface of the sheet of cells. 6 hours later, cells were washed with balanced salt solution and 5 ml of maintenance medium consisting of twice concentrated Eagle's basal medium and 1 per cent horse serum were added. Titration of virus infectious for eggs was carried out with aliquots of the fluid overlay removed at that time, and at daily intervals thereafter for 6 days.

The results of a typical experiment using strain swine 1976 are shown in Fig. 3. The level of virus increased from $10^{1.0}$ EID_{50} at 6 hours to approximately $10^{6.5}$ EID_{50} at 24 hours after inoculation. Until the 4th day the level of virus remained relatively constant, but by the 6th day, the amount of virus in the medium had declined.

Other strains of influenza virus, including the swine strain November 53, and four human isolates were also found capable of multiplying in cultures of epithelial cells derived from swine lungs. The data presented in Table II suggest that higher levels of virus were attained in this system by isolates obtained from swine than from humans. Perhaps swine isolates are somewhat better adapted for growth in cells of swine lung origin than are isolates from man. None of the strains employed produced detectable cytopathology or hemagglutinin, either at the 3rd or the 6th day following inoculation of virus. Six serial passages of swine strain 1976 were carried out in epithelial cell cultures. No increase in the yield of virus as measured in eggs was found, cytopathology did not become apparent, nor was the production of hemagglutinin observed.

TABLE II

Growth of Strains of Type A and B Influenza Virus in Epithelial Cells of Swine Lung

Virus Strains	Titer at 6 hours	Titer at 72 hours	Log increase in virus
Swine 1976	2.3*	5.9	3.6
Swine Nov. '53	2.5	6.5	4.0
PR8	3.5	5.5	2.0
AA/1/57	1.7	5.5	3.8
AA/23/57	1.5	4.3	2.8
Lee	1.5	4.0	2.5

* Log egg infectious dose per 0.1 ml.

However, Hinz and Syverton (25), using a different method for preparing sheets of cells, and different media for maintenance, were successful in demonstrating both the production of hemagglutinin and the development of cytopathology. In their preparations the yield of virus was higher than was attained in the present experiments. These findings lend encouragement to the possibility that cultures of lung cells *in vitro* might be employed to obtain further information about the hypothetical transmission cycle of swine influenza virus without incurring the expense of maintaining swine in isolation.

Fate of Infectious Virus Added to Minced Earthworms In Vitro and to Intact Earthworms.—A final phase of this study was an investigation of the capacity of earthworms to support multiplication of swine influenza.

Cultures of the Maitland type were prepared by mincing earthworms and by mincing organs in which lungworm larvae localize, *i.e.*, esophagus, calciferous glands, and heart. Each flask contained 10 to 12 fragments of about 1 mm in diameter, and 2.7 ml of the medium used for the cultures of lungworms. The cultures were inoculated by the addition of 0.3 ml of infected allantoic fluid containing 8.5 EID_{50} of swine strain 1976. Levels of virus in the supernate were measured in eggs using aliquots removed immediately after the addition of virus, at 24 hours, and at daily intervals up to 1 week.

An increase of virus in the supernate was not found. In other experiments, virus multiplication could not be shown when 10^2 EID_{50} were used for inoculation. Nor was prolonged survival of virus infectivity in the culture flasks observed, for the rate of decline of virus in the medium paralleled that of virus in flasks that did not contain fragments of earthworms.

Experiments using intact earthworms likewise gave negative results. Whole living earthworms were inoculated in the area of the esophagus with 0.1 ml of undiluted allantoic fluid containing $10^{8.5}$ EID_{50}. The earthworms were replaced in a moist soil and leaf environment. After 6 days, virus could not be recovered from a suspension prepared with the inoculated earthworms.

Thus, evidence that the earthworm supports virus multiplication was not found. That finding correlates with previously described failure to detect receptor substances in extracts of earthworms. Hence, the role of the earthworm in the proposed transmission cycle would seem at best to be passive, *i.e.*, that of an intermediate host for lungworms.

SUMMARY AND CONCLUSIONS

Swine lungworm extracts and suspensions of swine lungworms contain receptor-like substances capable of adsorbing influenza virus, a result consonant with the hypothesis (5–8) that the lungworm may be involved in the swine influenza cycle. Yet no evidence for multiplication of virus or even persistence of infectious virus in lungworms at undimished titer was found. Clearly much more information is needed, and it is hoped that the present demonstration of the practicality of studying the components of the transmission cycle proposed by Shope, will provide important tools requisite for further investigation of this problem.

Studies on the role of the earthworm in the transmission of swine influenza suggest that, at best, that role would be a passive one.

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