EPIZOOTIOLOGY OF SWINE INFLUENZA

By

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TITLE

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D.P.N.

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CHAPTER I

INTRODUCTION

Swine influenza is a relatively new disease of swine and has been traced back to human influenza pandemic of 1918 (Smith *et al.*, 1933). The complex etiology of clinical form of the disease in the field condition according to Shope (1931) and Lewis and Shope (1931) involves a viral and a bacterial component both compounding each other to produce the disease. The bacterial component is *Hemophilus influenzae suis* which is a small nonmotile, Gram negative, hemoglobinophilic bacillus. The swine influenza virus is a RNA-virus belonging to Myxovirus group, 80-120 mu in diameter (Elford *et al.*, 1936). It is identical in size and shape to human A influenza virus. Antigenically swine influenza virus is closely related to Influenza A virus. The virus is pathogenic for ferrets, white mice and swine (Andrews *et al.*, 1934; Shope, 1931, 1934). Inoculated alone intranasally the virus produces a mild febrile illness in pigs known as "filtrate disease" (Shope, 1931). Clinical form of swine influenza can be induced in pigs by experimental inoculation of both *H. influenzae suis* and swine influenza virus (Lewis and Shope, 1931).

Recent work by Underdahl (1958) and Nayak *et al.* (1962, 1964) and Wescott and Todd (1964) have indicated that a third component (helminths) might be involved in producing and enhancing the clinical form of swine influenza in the field. Experimentally prepatent and patent lungworms (*Metastrongylus spp.*) and migrating larvae of *Ascaris suum* can compound

an otherwise mild form of disease into a fulminating form with a clinical syndrome similar to that found field cases. In addition Shope (1941) and Sen et al. (1961) reported that migrating ascaris provoked the latent swine influenza virus in the lungworms and caused the outbreak of swine influenza epizootics in the field. Accordingly, a series of experiments have been devised to evaluate the synergistic relation between A. suum and swine influenza virus in enhancing the disease and to elucidate the mechanism of synergism.

Swine influenza virus was discovered and isolated by Shope in 1931 but only a few studies have been done on the pathogenesis of this agent in experimental and natural hosts (Shope, 1931, 1934; Dublin, 1945). Shope (1931) noted that virus caused a plum colored consolidation experimentally in the lungs of pigs and ferrets. The prominent microscopic lesions in the lungs were lobular emphysema and atalectasis with polymorphonuclear exudate in the bronchial lumen, round cell infiltration in the peribronchial and perivascular areas and thickening of alveolar septa. Dublin (1945) reported that early stages of infection in mice resulted in focal necrosis of the bronchial epithelium. This was followed by hyperplasia and regeneration with thickening of bronchial epithelium and alveolar septum. Hers et al. (1962) studied progressive infection of S 15 strain of swine influenza virus in mice by indirect immunofluorescent (IF) technique. Immunofluorescent techniques have not been used to follow the course of swine influenza infection in its natural host--swine. In the experiments to be reported here the progressive stages of development of swine influenza virus in mice and pigs have

been followed in sequence at the cellular level by using techniques of immunocytology and histopathology.

Shope (1941, 1943), Sen et al. (1961) and Kammer and Hanson (1962) have demonstrated that swine influenza virus can be carried by an intermediate host--swine lungworms (Metastrongylus spp.). Lungworms carry the virus in a masked form and the latent virus can be provoked sometimes to produce the clinical disease by stressing the pigs with physical, chemical or biological (bacteria or helminths) agents. However, the form of latent virus in the lungworm vector has not yet been determined. Moreover, even the existence of virus in lungworms has not been conclusively proven. Accordingly, a series of studies have been constructed in an attempt to detect the virus in a conclusive way in lungworm vectors. The investigations reported here deal with the epizootiology of swine influenza virus in the following three aspects:

1. Etiology and pathology of swine influenza (S 15): Progressive pathogenesis of swine influenza virus in mice and swine; effects of varied inocula on the distribution and progression of virus will be determined by IF and histopathological techniques.
2. Synergism between helminths and swine influenza virus: The effect of lung phase of A. suum on (a) enhanced pathogenesis of S 15 virus; (b) production of viral antigen and infectious particles; (c) localization of virus in the lungs when injected intravenously; (d) optimum time for synergism; (e) toxicity of excess H antigen, etc. will be

studied by coinfecting mice with A. suum and S 15 virus.

3. Swine lungworms as intermediate host for swine influenza virus: Attempts would be made to isolate the infective virus from extract of infective adult lungworms in mice and pigs.

MATERIALS AND METHODS

Experimental Animals:--a) Pigs - The pigs used in these experiments, if not otherwise specified, were procured by hysterectomy on the 112th day of gestation and maintained in isolation chambers without colostrum according to the method of Young, Underdahl and Hinz (1955) and Underdahl and Young (1957). The baby pigs were removed from their embryonic membranes while inside a sterile hood and transferred to individual modified Horsfall units. Each unit was attached to a negative air pressure system and had a filtered air inlet and outlet (Young and Underdahl, 1953). A diet of homogenized milk, egg, mineral mixture and vitamins was fed in stainless steel pans (Underdahl and Young, 1957). Temperature of the room and chambers was maintained at 35 C (95 F). As the pigs became older (usually one week of age), they were transferred to a larger "brooder" unit containing up to 12 pigs. Air supply of these chambers was also filtered but in these brooders the pigs received a continuous supply of dry feed and fresh water in addition to rations of milk. When the pigs reached one month of age they had adapted to dry feed and were usually transferred onto the floor of isolation rooms.

b) Mice - Swiss albino mice of five to six weeks were used. They came from a closed laboratory colony, bred and maintained in the Department of Veterinary Science, University of Nebraska. The usual care and hygienic conditions of the mice were good. Mortality rate among the baby mice was insignificant and repeated histological examination did not show pulmonary involvement. Mice were weaned when four weeks of age. Experimental mice were isolated from breeding colonies.

c) Chicken embryo - Fertile eggs were incubated at 37.8 C (100 F) in a humid chamber for 10 days. They were turned twice a day. The development of embryo was periodically checked by candling during the incubation period. When 10 days old the living embryos were used for experiments.

Virus:--Egg and mouse adapted Shope's (S 15) strain of swine influenza virus were used in these experiments. The virus has been passaged 232 times in mice and 26 times in chicken embryo. Seed virus was prepared from infective egg allantoic fluid harvested 48 hours post-inoculation, sealed in one ml ampules and stored at -60 C. The stock virus was found to contain 320 hemagglutinin units (HA), $10^{7.7}$ EID_{50} (50% egg infective units) per ml (Reed and Munech, 1938).

Embryonation of Ascaris Eggs:--Living worms (Ascaris suum) were collected from a local abattoir; uteri were dissected out, homogenized in physiological saline. Eggs were washed several times by centrifugation and resuspension in saline solution. The eggs were then decoated in chlorox solution (2.6% sodium hypochlorite) for 10-15 minutes in a magnetic stirrer. Decoated eggs were washed twice with distilled water by centrifugation and finally suspended in 0.1 N H_2SO_4. Twenty-five to thirty milliliters of the suspended eggs were distributed in 125 ml Erlenmeyer flasks and incubated at 30 C for three weeks with constant aeration (Kelley and Nayak, 1964). The development of the egg embryos was checked periodically. Infectivity of embryonated eggs was checked in mice before using in experiment. The embryonated eggs were kept at 4 C and the required doses were prepared by dilution count from stock egg culture.

Virus Inoculation:--Mice were inoculated intranasally with 0.015 ml (2 drops from a 27 gauge needle) of infective material while they were under light ether anesthesia. The infective material was prepared by dilution of stock egg allantoic fluid containing virus with nutrient broth or phosphate buffered saline.

Pigs were also inoculated intranasally with the virus inoculum while they were under ether anesthesia. The inoculum consisted of 0.5 ml of infective allantoic fluid containing 160 HA units diluted to 2 ml with nutrient broth.

Procedure of Necropsy:--The pigs were bled to death by severing the brachial arteries while under sodium pentobarbital anesthesia. After death, the chest cavity was opened, the lungs were removed and examined for gross lesions. Spleen, liver, kidney were also examined and portions of tissues from these organs as well as lungs were saved for histology, immunofluorescent study or for virus isolation. Mice were killed by ether or chloroform and immediately after the organs were dissected out.

Counting Larvae:--Five mouse lungs were pooled, homogenized with tap water and the volume was adjusted to 10 ml. Five 0.5 milliliter aliquots were counted after proper stirring. The number of larvae per lungs was calculated from the dilution factor.

Production Swine Influenza Antibody:--Three one-month old specific pathogen free (SPF) pigs were inoculated intranasally-orally with 5 x $10^{6.74}$ EID_{50} units of virus while under light ether anesthesia. All three developed high fever, dyspnea and anorexia within 24-48 hours

following the inoculation of virus. A second inoculation was given 25 days following the initial one using the same dose and techniques. The reaction following this second inoculation was mild resulting in only a slight fever. The third inoculation of virus ($5 \times 10^{6.74}$ EID_{50}) was given intraperitoneally on the 60th day after the first inoculation. The fourth inoculation consisted of the same amount of virus given intramuscularly on the 90th day. Finally all the pigs were inoculated intramuscularly with 3.5 ml concentrated allantoic fluid virus on the 115th day ($3.5 \times 10^{7.74}$ EID_{50}). No clinical reaction could be detected following the third and subsequent inoculation. The pigs were bled following virus inoculations and hemagglutination inhibition (HI) titer and gamma globulin concentration was determined. Pigs were exsanguinated 15 days following the last inoculation. At this time the average titer of pooled serum was 1:10240 HI units per ml. Hemagglutination (HA), hemagglutination inhibition (HI), egg infectivity (EID_{50}) and mouse infectivity (MID_{50}) were done according to standard procedures.

Fractionation of Globulins:--Globulins were fractionated from the whole serum by dialyzing at 4 C overnight against ammonium sulfate solution in final concentration 1.39-1.50 M while being constantly stirred. The precipitate was centrifuged, washed with 1.50 M ammonium sulfate solution and dissolved in 0.05 M sodium carbonate-bicarbonate-saline (pH 9). To remove the sulfate ions, the globulin was dialyzed for four days at 4 C against the above buffer which was frequently changed. Paper electrophoresis indicated that the fraction contained 60 per cent gamma, 17 per cent beta and 18 per cent alpha globulins and 5 per cent albumin.

HI titration indicated that 35 per cent of total antibody had been recovered by this method.

Conjugation of Globulin:--Crystalline fluorescein iso thiocyanate[1] (FITC) 12-15 mg/gm was dissolved in several drops of cold acetone and 5 ml of 0.05 M sodium carbonate-bicarbonate-saline buffer (pH 9) were added. The globuline fraction was added to the fluorescein solution and total volume was adjusted with the above buffer to make a final concentrate containing 2 per cent protein. This FITC-globulin mixture was mixed well, and kept at 4 C without stirring for 24 hours. The solution was then dialyzed in PO_4 buffered saline solution (0.05 M) with 20 gm of AG2-X4 20-40 mesh,[2] an ion exchanger (chloride form), and later for 48 hr in the same buffer without ion-exchanger, buffer was changed frequently. The free fluorescein was presumed to be removed when the buffer was no longer fluorescent under Wood's UV lamp.

Treatment of the Conjugate:--The dialyzed conjugate was centrifuged to remove the precipitate and treated by any of the following three methods:

1. Absorbed by swine liver powder thrice as described by Coons et al. (1950) and Kaplan et al. (1950).
2. After treatment with swine liver powder the conjugate was fractionated in a Diethyl-aminoethyl (DEAE)[3] cellulose

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[1]Lot No. 1036, Sylvania Chemical Company, Orange, New Jersey.

[2]Bio-Rad Laboratories, Richmond, California.

[3]Type 20, Brown Company, Boston, Massachusetts.

chromatography by a linear salt gradient (0.01 M to 1 M NaCl) according to Goldstein et al. (1962).

3. A third portion was only fractionated by DEAE cellulose column chromatography without any pre-treatment of liver powder.

Fluorescein-protein ratio was determined by absorption at 280 mu and 495 mu on a Beckman Model DB spectrophotometer (Golstein et al., 1962). Protein concentration was likewise determined by absorption spectrophotometry as well as by Lowry's method (1951). All the conjugates were dialyzed in 0.01 M phosphate buffered saline before using for staining. A dilution test was run on each conjugate to determine the endpoint of brilliant specific fluorescence. HI titration was done simultaneously and it was found that 160 to 320 HI units were necessary for specific immunofluorescent (IF) reaction with this conjugate.

Sectioning and Staining:--Fresh tissue was collected immediately after the animal was killed, frozen quickly in dry ice and stored at -20 C. Sections were cut 4 to 6 u in a cryostat. In the beginning of this investigation the sections were stained with conjugate plus complement as reported previously (Stair et al., 1963) but later it was found that complement was not needed for this system and the alternate method used was as follows:

The sections were fixed in chilled acetone for 10 min at 4 C, dried in incubator at 37 C, rinsed with phosphate buffered saline solution (0.01 M, pH 7) and overlaid with conjugate. These preparations were incubated in moist chamber at 37 C for 40 min. At the end of the