SYNERGISTIC ACTION OF HEMOPHILUS INFLUENZAE SUIS AND THE SWINE INFLUENZA VIRUS ON THE CHICK EMBRYO. II

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Swine influenza is caused by two agents, a bacterium (*Hemophilus influenzae suis*) and the virus of swine influenza, acting in concert to produce an extensive pneumonia (1). This synergistic action can be reproduced by inoculating the two agents on the chorioallantoic membrane of 9 to 10 day chick embryos, thus rapidly killing a high proportion of embryos and causing selective destruction of the lung tissue in those surviving (2). This effect is not produced by the action of either virus or bacterium alone.

Bacteria are not seen in sections of these lungs, and heart blood cultures from embryos killed during the peak of mortality show no growth (Table I). However, since *H. influenzae suis* persists on the chorioallantoic membrane of embryos infected with swine influenza virus longer than on normal embryos, the bacterium apparently benefits by the presence of the virus. Embryos dying early show the same generalized hemorrhages and later cell destruction seen in the occasional embryo killed by virus alone. For these reasons it is unlikely that the death of the embryos receiving the two agents is accompanied by invasion of the tissues by *Hemophilus*. The complementary problem of the effect of the bacterium on the virus will be considered in this paper.

Method

As in the previous study (2), 9 day embryos were inoculated with virus. Blood containing *H. influenzae suis* was added the next day. Again the mortality figures refer to the percentage of embryos dead 48 hours after inoculation with *Hemophilus* preparations. Pooled material from several embryos which had received identical inoculations was titrated, so that the results represent an average of the particular group of embryos tested.

Titrations for virus were carried out by two different techniques: (1) 1 drop of each serial tenfold dilution of the test material was inoculated on the chorioallantoic membrane of at least three 9 to 10 day embryos. These embryos were incubated for 48 hours and then tested for the presence of virus by the clumping of the chick's own red blood cells (3). The theoretical point at which 50 per cent of the embryos were infected was calculated and used as the end-point (4). (2) Other titrations were made by intranasal inoculation of 3 to 5 weeks old mice under light ether anesthesia, each dilution being inoculated into 3 mice, and an arbitrarily weighted 50 per cent end-point was calculated from the data collected during 10 days' observation and at final autopsy.

RESULTS

The first titrations showed that there was much more virus in the allantoic fluid of those embryos which received the combination of virus and bacterium, but that the virus in the membrane itself had not increased (Table II). For this reason, membranes, allantoic fluid, and embryos were tested in the same

TABLE I

Cultures of Embryos Infected with Swine Influenza Virus and Hemophilus influenzae suis

Hours after inoculation with *Hemophilus*	Heart blood
5	–*
10	– – –
19	– – – –
31	– – – –
61	– –

* Each sign represents an individual embryo which was cultured and studied histologically.

TABLE II

The Effect of Hemophilus influenzae suis on the Spread of the Swine Influenza Virus through the Embryonated Egg

Material titered	No. of embryos sampled	Titered in	Titer	
			Virus alone	Virus + *Hemophilus*
Allantoic fluid, 31 hrs.	3	Embryos	$10^{-3.7}$	$10^{-7.2}$
Allantoic fluid, 61 hrs.	2	Embryos	$<10^{-2.5}$	$10^{-4.3}$
Membrane, 24 hrs.	2	Mice	$10^{-4.3}$	$10^{-3.9}$
Membrane, 60 hrs.	3	Embryos	$10^{-5.3}$	$10^{-6.0}$
Membrane, 16 hrs.	2	Mice	$10^{-5.0}$	$10^{-3.8}$
Allantoic fluid, 16 hrs.	2	"	$10^{-2.0}$	$10^{-4.2}$
Embryo, 16 hrs.	2	"	$10^{-0.9}$	$10^{-3.3}$

* Titrations done on separate experimental series are divided by horizontal lines.

series, and again they showed the same amount of virus in the membranes but much more in the allantoic fluid and embryos of the series which had received the combination. These findings indicate that the bacteria cause the virus to spread from the membrane into the allantoic fluid.

The synergistic effect of other bacteria was studied briefly. A strain of *Pasteurella* recently isolated from pigs and a gonococcus were tested. The former was chosen because it has been suggested that, instead of *Hemophilus*,

Pasteurella may sometimes act with swine influenza virus to produce the complex disease. The gonococcus was chosen because it was totally unrelated to the problem and because it was known to kill only a few embryos when used before embryo adaptation. Both of these organisms killed only a few more embryos infected with swine influenza virus than would be expected if the two agents act separately (Table III).

All of these data are consistent with the idea that a "spreading factor" like that obtained originally from testicle extracts (5) and from the pneumococcus (6) and other bacteria is responsible for the spread of the virus through the embryo. Such a factor can spread a number of viruses through susceptible tissues (7) and thus enhance the disease processes. Indeed it has been sug-

TABLE III

The Effect of the Combination of Other Bacteria and Swine Influenza Virus on Mortality

Organism	No. of embryos					
	Virus alone		Virus + bacteria		Bacteria alone	
	Dead	Alive	Dead	Alive	Dead	Alive
Pasteurella	4	8	4	9	1	10
"	1	11	8	5	3	9
Gonococcus	2	6	8	1	2	7
"	2	4	4	2	1	4
Total	9	29	24	17	7	30
Mortality, *per cent*	24		59		19	

gested that this factor is responsible for the enhancing action of *H. influenzae suis* on the influenza virus in the pig (8, 6).

A purified preparation of hyaluronidase, which is at least closely related to if not identical with the spreading factor elaborated by the pneumococcus (9), was obtained through the courtesy of Dr. Karl Meyer, of the College of Physicians and Surgeons, Columbia University. Three to 4 drops of saline containing varying amounts of this material (see Table V) were added to the membranes of 10 day embryos which had been infected the previous day with the swine influenza virus; the membrane and allantoic fluid were later titered for virus content. In 2 of 3 experiments the virus in the allantoic fluid of these embryos was present in higher concentration than that in the controls (Table IV). A significant increase was not demonstrated in the third series, but in this experiment, using embryo-adapted virus, most of the controls inoculated with virus alone died rapidly.

It is noted that hyaluronidase did not increase the mortality of influenza virus embryos (Table V) as effectively as did cultures of *Hemophilus* (2).

Mortality in the latter case frequently increased from below 10 per cent when virus alone was used to 60 per cent or more when the combination was given. However, this work was done with 24 hour cultures of *Hemophilus* in 0.5 cc. of blood at the base of an agar slant. In order to eliminate any effect of the

TABLE IV

The Effect of Hyaluronidase on the Spread of Swine Influenza Virus in the Embryo

Material titered	No. of embryos sampled	Titered in	Titer	
			Virus alone	Virus + hyaluronidase
Membrane, 48 hrs	2	Mice	$10^{-4.3}$	$10^{-4.5}$
Allantoic fluid, 48 hrs	2	"	$10^{-1.2}$	$10^{-5.3}$
Membrane, 48 hrs	3	Embryos	$10^{-6.7}$	$10^{-7.0}$
Allantoic fluid, 48 hrs	3	"	$10^{-4.0}$	$10^{-6.5}$
Membrane, 24 hrs	2	Mice	$10^{-4.2}$	$10^{-0.5}$
Allantoic fluid, 24 hrs	2	"	$10^{-4.2}$	$10^{-5.0}$

Compare with Table II.

TABLE V

The Effect of Hyaluronidase on the Mortality of Embryos Infected with Swine Influenza Virus

Amount of hyaluronidase	No. of embryos					
	Virus alone		Virus + hyaluronidase		Hyaluronidase alone	
	Dead	Alive	Dead	Alive	Dead	Alive
mg.						
0.7	1	6	3	6	0	5
0.3	7	2	8	2	0	8
0.4	2	6	4	2	—	—
0.2	3	8	4	8	0	7
Total	13	22	19	18	0	20
Mortality, *per cent*	37		51		0	

blood, experiments were then performed with 24 or 48 hour growths of *Hemophilus* on chocolate agar slants.

The growth from several slants was suspended in saline. The bacteria were killed by heating in a closed glass tube submerged in a water bath for ½ hour. The dead bacteria can be thrown down by centrifuging for 10 minutes at 8000 R.P.M. in a Pickels centrifuge.

A fresh preparation of these dead bacteria readily kills embryos infected with swine influenza virus. An extract of bacteria prepared by freezing, drying and grinding, resuspending in saline, and filtering through a Berkefeld candle, also kills embryos infected by swine influenza (Table VI).

The strength of the killed bacterial suspension may be tested by inoculating embryos with serial fourfold dilutions (Table VII). One drop of a suspension

TABLE VI

The Effect of Extracts and Killed Hemophilus influenzae suis on the Mortality of Embryos Infected with Swine Influenza Virus

Type of *Hemophilus* preparation	No. of embryos					
	Virus alone		Virus + *Hemophilus*		*Hemophilus* alone	
	Dead	Alive	Dead	Alive	Dead	Alive
Saline extract of frozen, dried bacteria	1	8	6	4	1	6
Supernatant from suspension of killed bacteria	2	6	3	5	—	—
Killed bacteria	2	6	7	1	—	—

TABLE VII

Titration of Suspension of Killed Hemophilus on 10 Day Embryos Infected with Swine Influenza Virus

	No. of embryos infected with virus + 1 drop of:							
	Original suspension		1/4 of original suspension		1/16 of original suspension		Saline	
	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive
	11	4	8	7	3	12	1	14
			9	8	5	12	0	17
Total	11	4	17	15	8	24	1	31
Mortality, *per cent*	73		53		25		3	

This table is based only on embryos killed within 24 hours after the addition of the *Hemophilus* suspension; 48 hours afterward the 1/16 dilution showed 56 per cent mortality.

containing less than 0.2 mg. protein per cc. (calculated from the protein nitrogen) killed 25 per cent of the embryos in 24 hours and 56 per cent in 48 hours (Table VIII). This result may be compared with those obtained by inoculating 3 to 4 drops of the purified hyaluronidase preparation at a concentration of 0.8 mg. of the purified preparation per cc. (Table V). By simple calculation we find that the same slight increase in mortality produced by hyaluronidase can be reproduced by about 1/16 the amount of a suspension of killed *Hemophilus*.

A further difference between the *Hemophilus* preparation and the hyaluronidase is demonstrated by the effect of heat. Hyaluronidase and the pneumococcus spreading factor are weakened by heating at 60°C. and almost entirely destroyed by 100° for 30 minutes (9). These temperatures fail to affect the mortality produced by preparations of *Hemophilus* (Table IX).

TABLE VIII

The Effect of a Dilute Suspension of Hemophilus on the Mortality of Embryos Infected with Swine Influenza Virus

	No. of embryos			
	Virus alone		Virus + *Hemophilus*	
	Dead	Alive	Dead	Alive
	6	10	8	7
	1	16	10	7
Total.....	7	26	18	14
Mortality, *per cent*.....	21		56	

Compare with Table V in which a hyaluronidase preparation of 16 times this protein concentration produced the same mortality.

TABLE IX

The Effect of Heated Suspensions of Hemophilus on the Mortality of Embryos Infected with Swine Influenza Virus

	No. of embryos							
	Virus alone		Virus + bacterial suspension heated 30 min. at					
			50°C.		70°C.		100°C.	
	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive
	1	8	5	6	6	3	7	4
Mortality, *per cent*.....	11		45		67		63	

DISCUSSION

Knowledge of the mechanism of the reaction between the embryo host and the bacterium and virus of swine influenza remains incomplete, yet it may be worth while to outline the known facts and suggest a hypothesis for future work. The infection of the membrane by swine influenza virus allows the *Hemophilus* to persist (2). The addition of live or killed bacteria or of bacterial extracts greatly increases the mortality of embryos infected with swine influenza virus. Embryos that survive the immediate high mortality after inoculation with the two agents frequently show a selective destruction of lung tissue but

no bacteria in the embryo. The bacteria in turn do not raise the virus titer locally but enable it to spread through the membrane into the allantoic fluid. Such spread is also furthered by hyaluronidase; but this by no means indicates that the bacteria act synergistically with the virus because of hyaluronidase, since there may be a variety of spreading factors (9).

In analyzing the synergistic effect of *H. influenzae suis* and the swine influenza virus, we must clearly differentiate between the early killing effect and the later selective destruction of the lung tissue of the embryo. In the first case synergism is not manifested alone by the spread of the virus from the membrane to the embryo for three reasons: (1) The quantitative relations of hyaluronidase and killed cultures of *Hemophilus* are not compatible with the idea that the killing effect of the bacteria is due to spread, since the very slight effect of hyaluronidase can be reproduced by 1/16 that amount of *Hemophilus* culture as measured by total nitrogen. (2) The spreading effect of hyaluronidase is easily destroyed by heat, but cultures of *Hemophilus* retain their ability to kill influenza embryos even after heating at 100°C. for 30 minutes. (3) Intra-amniotic injection of the embryo with virus alone does not strikingly increase the mortality. On the other hand, selective destruction of the lung tissue does occur following intra-amniotic injection of virus alone. The similarities or dissimilarities of the pneumonia produced by virus alone and by virus plus *Hemophilus* can only be determined by a larger series of sections of embryos in all stages of infection.

SUMMARY AND CONCLUSIONS

Blood cultures of embryos killed by the synergistic action of swine influenza virus and *Hemophilus influenzae suis* are consistently negative, and embryos infected with swine influenza virus may be killed both by filtered extracts of frozen and dried *Hemophilus* and by suspensions of heat-killed bacteria. The addition of *Hemophilus* to the chorioallantoic membrane of embryos infected with swine influenza virus causes the virus to spread from the membrane to the allantoic fluid and embryo. This spreading effect also obtains when a purified preparation of hyaluronidase is used instead of *Hemophilus*, but it is unaccompanied by a comparable increase in mortality. It is probable that the spread of the virus produced by the bacteria is only partly responsible for the development of the complex infection and that products of these organisms other than the spreading factor play a large part in the mortality of embryos receiving the combination of virus and bacterium.

BIBLIOGRAPHY

1. Shope, R. E., *J. Exp. Med.*, 1931, **54**, 373.
2. Bang, F. B., *J. Exp. Med.*, 1943, **77**, 7.
3. Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

4. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27,** 493.
5. Duran-Reynals, F., *J. Exp. Med.*, 1929, **50,** 327.
6. Duran-Reynals, F., *J. Exp. Med.*, 1933, **58,** 161.
7. Hoffman, D. C., *J. Exp. Med.*, 1931, **53,** 43.
8. Shope, R. E., *Harvey Lectures*, 1935–36, **31,** 183.
9. Meyer, K., Hobby, G. L., Chaffee, E., and Dawson, M. H., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44,** 294; *J. Exp. Med.*, 1941, **73,** 109.