THE INFLUENCE OF INFLUENZA VIRUS INFECTION ON EXOGENOUS STAPHYLOCOCCAL AND ENDOGENOUS MURINE BACTERIAL INFECTION OF THE BRONCHOPULMONARY TISSUES OF MICE*

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It is well recognized that representatives of three bacterial genera, *Staphylococcus*, *Streptococcus*, and *Hemophilus*, which are frequently present in the upper respiratory tract of man, have only a limited capacity to produce a primary pneumonia in adults. When pneumonia induced by one of these microorganisms does develop in an adult, it arises almost invariably in association with influenza virus infection or preexisting morphologic abnormalities of the bronchopulmonary structures. This situation is quite unlike that seen with *Pneumococcus* which has the capacity to initiate a primary pneumonia in the absence of influenza viral infection.

The mechanism by which influenza influences staphylococcal, streptococcal, or influenza bacillus pneumonia has been the subject of relatively few studies in laboratory animals in the 28 years that have elapsed since the first isolation of influenza virus. Human studies have usually not employed the methods of quantitative bacteriology and have been complicated by the widespread use of antimicrobial agents.

In 1941, Glover (1) noted the spontaneous occurrence of Group C streptococcal pneumonia in ferrets infected with influenza A virus. He demonstrated the increased

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pathogenicity of this combination and the inability of streptococci to induce the disease in the absence of the viral infection. Later, Francis and DeTorregrosa (2) found that strains of *H. influenzae* produced extensive lethal pneumonia in mice previously infected with influenza virus. It was necessary for influenza virus infection to precede the introduction of bacteria; simultaneous inoculation of the two agents or of *H. influenzae* alone resulted in no bacterial disease. Similar results were noted with strains of beta hemolytic streptococci. Carlisle and Hudson (3, 4), also demonstrated the synergism of a combination of beta hemolytic streptococci and influenza A virus.

Harford and others (5) observed that pneumococci introduced intrabronchially were cleared from the normal mouse lung with great efficiency. Even when influenza virus had been present 24 hours and multiplication of virus had begun, disappearance of the pneumococci was not impeded. When pneumococci were introduced after 5 days of influenza, however, they multiplied and produced extensive disease. This suggested the operation of some factor concerned with the influenza viral lesion which did not appear until after more than 24 hours of viral infection. Harford considered this factor to be pulmonary edema fluid and has reported a number of experiments which support this concept. (6).

Gerone *et al.* (7), employing aerosols of pneumococci, were able to demonstrate synergism between these bacteria and influenza A virus in terms of greatly increased mortality of mice and multiplication of pneumococci in the lung. This was the case, however, only when the pneumococci were introduced 6 days or more after the initiation of viral infection.

Verlinde and Makstenieks (8) have reported that staphylococci produced only limited foci of leukocytic infiltration in normal monkeys after intratracheal or intranasal inoculation. When influenza A virus was given alone, evidence of bronchiolar necrosis and desquamation appeared, together with intra-alveolar and peribronchiolar edema. When the monkeys were infected with both agents, more extensive bronchiolar necrosis and inflammation occurred with extension of the process to involve the entire respiratory tract. None of the animals developed lesions extensive enough to cause death. These experiments were not designed to examine any aspects of the relative timing of the two infections, nor were quantitative bacterial studies made.

Two features characterize the results in all of these studies. (*a*) The four microbial genera employed all failed to produce disease in the lungs of normal laboratory animals. Nevertheless, in animals in which influenza viral lesions had reached a certain stage of evolution, any one of the four microbial agents produced bronchopulmonary disease. (*b*) In all of these earlier studies the strains of influenza virus used had received passage in laboratory mammals and hence were relatively pathogenic or "adapted" to mice.

Most of the investigations cited had to do with pneumococci. Only in the studies of Francis and DeTorregrosa (2) and Verlinde and Makstenieks (8) were staphylococci employed. This relative dearth of information on influenza virus–staphylococcal relationships is particularly important today because the staphylococci are probably second only to pneumococci as inducers of post-influenza bacterial pneumonia (9, 10). Moreover, the development of staphylo-

coccal pneumonia in the adult human is frequently dependent on prior infection with influenza virus.

In addition, all of the above studies were concerned with the effect of infection with influenza virus on the consequences of subsequent exogenously induced bacterial infection. There is very little experimental information regarding the effects of influenza virus on the relationship of the host with *preexisting* bacteria within the bronchopulmonary tissues.

Accordingly, the present studies were undertaken with two objectives in mind; (*a*) to assess quantitatively the effects of influenza virus infection upon the mechanisms by which the host normally eliminates bacteria, specifically staphylococci, from the bronchopulmonary tissues, and (*b*) to inquire into the possible role played by latent endogenous bacteria of these tissues in the pathogenesis of influenza. The virus strain employed had not been subjected to previous murine passage and produced gross pneumonic lesions only infrequently when inoculated in high concentration. The non-fatal disease thus induced more closely resembles human infection with influenza virus than the more severe infection induced with mouse-adapted virus in earlier studies.

Materials and Methods

Influenza Virus.—The influenza virus used for all experiments was the Japan 305 strain of influenza A virus, (A/Japan/305/57) maintained by serial egg passage and not previously passaged in mice. The initial concentration of infective virus particles (EID_{50}, or egg infectious dose 50 per cent) was 10^8/ml. A 10^{-1} dilution of infected allantoic fluid in 0.85 per cent NaCl buffered to pH 7.1 with 0.01 M phosphate buffer solution constituted the viral inoculum. Approximately 0.04 ml of the diluted viral suspension was inoculated intranasally in each mouse, providing an infective dose of about $10^{5.7}$/mouse. This inoculum was prepared without antimicrobial drugs and was used immediately after dilution.

Staphylococcus Aureus.—Two coagulase-positive, hemolytic strains of *Staphylococcus aureus* were used. The Giorgio strain, phage type 47/53/56/75/77/VA4, is inhibited by penicillin at 3.1 μg per ml and by streptomycin at 0.4 μg per ml. This strain, when injected intravenously into mice, causes extensive renal abscesses and death of all animals if untreated. The Stovall strain is phage type 47/53 and is inhibited by penicillin at 25 μg per ml. It is resistant to more than 50 μg per ml of streptomycin, tetracycline, chloramphenicol, and erythromycin. It also causes death in 100 per cent of mice following intravenous inoculation and the animals dying of this infection show abscesses in many organs. Both strains were originally isolated from human infections.

Inocula.—The staphylococci used for intranasal instillation were prepared from an 18 hour nutrient broth culture. After centrifugation the microorganisms were resuspended in buffered saline. The 0.04 ml inoculum contained 2×10^7 viable staphylococci. Similar procedures were used in preparation of the suspension of staphylococci used for aerosol instillation, except that distilled water was used in place of buffered saline as the vehicle. Intravenous inocula were prepared by diluting the 18 hour culture 1:2 or 1:20 with saline, depending upon the final concentration required.

Mice.—18 to 20 gm male Swiss albino mice of the CFW strain were used. These were housed in wire cages, 10 animals per cage, and supplied with standard mouse pellets and water *ad libitum*.

Intranasal Inoculation.—The animals were lightly anesthetized with ether and three drops of infecting material from a 27 gauge needle (approximately 0.04 ml) were allowed to fall on the nose of each mouse. Influenza virus was introduced in this manner in all experiments and staphylococci in some as indicated.

Aerosol Procedure.—A distilled water suspension of staphylococci was aerosolized in the apparatus described by Middlebrook (11). Both control and experimental animals were exposed simultaneously for a period of 1 hour. No information is available concerning the size or concentrations of particles produced by this machine and therefore the actual infection dose of staphylococci is unknown. Middlebrook estimated that animals exposed for 1 hour to an aerosol of an original suspension of 1.5×10^6 tubercle bacilli received approximately 10,000 microorganisms per animal.

Tissue Homogenization.—Groups of surviving animals were autopsied at predetermined intervals. Lungs and other tissues to be examined were removed and homogenized, utilizing a modification of the Fenner-Martin-Pierce tissue homogenization technique (12, 13). The number of culturable staphylococcal units present in each organ was calculated and is expressed in all tables and graphs as the logarithm to the base 10.

TABLE I

Titers of Influenza Virus (Japan 305) in Lungs of Mice at Various Intervals After Inoculation of $10^{5.7}$ EID_{50} of Virus

After inoculation	EID_{50}/0.1 gm of lung
days	
1	$10^{-5.7}$
3	$10^{-4.8}$
6	$10^{-2.0}$
9	$<10^{-1}$
13	$<10^{-1}$

Titration of Influenza Virus in Mouse Lungs.—CFW mice (5/group) averaging 18.2 gm in weight were inoculated intranasally while under light ether anesthesia with 0.04 ml of a 10^{-1} dilution in phosphate-buffered saline of allantoic fluid containing $10^{8.0}$ EID_{50}/ml of the Japan 305 strain of influenza A virus. At the indicated time intervals lungs were removed with aseptic precautions, pooled in groups of 5, and ground in a high speed mixer (Lourdes) at 100 per cent speed for 3 minutes to make a 10 per cent suspension by weight. After centrifugation at 8,200 *g* for 5 minutes, supernatant fluid was removed. Titration of viral infectivity was effected in 10-day-old chicken embryos by the inoculation of serial 10-fold dilutions of the suspension by the allantoic route (4 embryos/dilution). Allantoic fluids were collected after 45 hours of incubation and individual fluids were tested at 1:4 dilution for the presence of hemagglutinin by the addition of a 0.5 per cent suspension of human "O" erythrocytes.

Pathology.—Mice were killed by cervical fracture. Lungs and heart were removed as a unit and fixed in formaldehyde. 6 randomized paraffin-embedded sections from each animal were stained with hematoxylin and eosin for section and later microscopic study.

Streak Culture.—In some experiments in which the bulk of the ground lung tissue was used for virus titrations, loop specimens were streaked on veal agar plates to obtain semiquantitative information regarding the numbers and types of bacterial organisms present.

EXPERIMENTAL OBSERVATIONS

The Course of Uncomplicated Influenza Virus Infection

In order to minimize variation in the extent of influenza from mouse to mouse, and to infect simultaneously as many respiratory tract cells as possible, a large dose of virus (an estimated $10^{5.4}$ EID_{50}), was inoculated in all experiments. That accelerated and short-lived infection was in fact achieved is illustrated by the fact that peak viral concentrations were attained within 24 hours of infection and infectivity titers subsequently declined progressively to the point of undetectability by the 9th day (Table I). Details of this experiment are presented above in Materials and Methods. In this experiment, gross pulmonary lesions were noted in only 1 animal and then on the 13th day. No deaths occurred.

Introduction of Staphylococci by Direct Intranasal Inoculation

It was the purpose of the first experiment to determine the optimal interval between viral and bacterial inoculation for induction of the maximal effect upon the fate of staphylococci introduced by direct intranasal inoculation.

At intervals respectively of 7 days, 4 days, 2 days, and 1 day before staphylococcal challenge, four groups of mice were infected with influenza virus and 4 other groups received buffered saline. On the day of challenge all animals received intranasal staphylococci. 3 animals from each pair of influenza and control subgroups were killed at 3 days, 5 days, 7 days, 11 days, 14 days, and 18 days following the staphylococcal challenge. Only the lungs were homogenized for microbial studies although the other organs were inspected for gross abnormalities.

Gross lesions were occasionally observed in the lungs of animals infected with influenza virus. These consisted of localized areas of plum-colored consolidation of portions of the lungs varying in size from subsegments to lobes, and at times involved an entire lung. Deaths were rarely observed in animals which received influenza virus alone. With the occasional exception of localized purulent abscesses, no gross evidence of the presence of staphylococci was discernible.

The populations of staphylococci in the lungs of the animals are presented in Table II. In the animals that received staphylococci after only 1 day of influenzal infection, the numbers of staphylococci in the lungs remained essentially constant for the ensuing 7 days and then were markedly diminished. Thereafter only an occasional animal showed evidence of persisting staphylococci; bacterial increase did not usually occur although there were exceptions which will be discussed later. When the influenzal infection had been present for 2 days before introduction of the staphylococci, the census of staphylococci remained constant and consistent for 5 days thereafter. Some of the animals sampled 7 and 11 days after the staphylococcal challenge, showed persistent, even increased, titers of bacteria, but these were a mixture of staphylococci with another organism which will be discussed below. Subsequently, only the

TABLE II

Numbers (Log 10) of Culturable Staphylococci Recovered from Lung Homogenates of Mice Inoculated Intranasally with Staphylococci at Various Intervals after Influenza Virus Infection or Buffered Normal Saline Control

Interval between influenza virus* and staphylococcal infection‡	Initial inoculum	Days after intranasal staphylococcal challenge					
		3	5	7	11	14	18
1 day	Influenza virus	3.59	3.04	2.74	7.51	0.74	0
		3.35	4.98	3.58	0.73	0	0
		3.13	4.07	2.03	0	0	0
	Saline	1.33	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
2 days	Influenza virus	4.55	3.89	3.21	5.44	1.63	0.73 0
		2.60	4.64	0	5.75	0	0 0
		3.34	5.10	0	0	0	0 0
	Saline	2.13	1.89	0	0	—	—
		0	0	0	0	—	—
		0	0	0	0	—	—
4 days	Influenza virus	3.24	6.36	0	0	1.96	0 0
		3.38	6.45	0	0	0	0 0
		5.10	3.06	0	0	0	0 0
							0
	Saline	1.05	0	0	0	—	—
		0.74	0	0	0	—	—
		0	0	0	0	—	—
7 days	Influenza virus	0	0.73	2.40	0	0	1.21
		0	0	1.75	0	0	0
		0	0	0	0	0	—
	Saline	1.72	4.52	0	0	—	—
		0	4.13	0	0	—	—
		0	0	0	0	—	—

* Non–mouse-adapted A/Japan/305/57 virus. EID_{50} $10^{5.7}$ virus particles.

‡ Giorgio strain of staphylococcus.

sporadic recovery of small numbers of staphylococci was noted. When the staphylococci were introduced after 4 days of viral infection, they clearly did not persist beyond the 5th day of observation (9 days after influenza infection). Some of the individual counts suggested that multiplication had occurred to some extent by the 5th day, but subsequently this trend did not continue.

When the influenzal viral infection preceded the staphylococcal challenge by 7 days, no evidence of significant persistence of the staphylococci was obtained. Instead, the populations of staphylococci disappeared from the lungs at the same rapid rate noted in the control animals.

At no time was persistence of staphylococci noted in the control animals, which had received saline instead of influenza virus. The staphylococci were detected for successively shorter periods in groups of animals with influenza viral infections of longer duration. Thus, unlike the situation in the control animals, the introduction of staphylococci at various times in relation to the viral infection resulted in an essentially constant census of staphylococci which was maintained until the viral infection was approximately 7 to 10 days old and by which time infective virus had disappeared. After this point in the evolution of the viral infection, freshly inoculated or previously established staphylococci disappeared from the bronchopulmonary tissues almost as rapidly as they had in the control animals that had received no virus.

Introduction of Staphylococci by Aerosol Inhalation

In an effort to produce more consistent staphylococcal lesions in the lungs, mice were infected with influenza virus by direct intranasal inoculation and 3 days later were exposed to an aerosol mist containing staphylococci. Although the number of infectious particles inhaled by each mouse was not determined, control observations 15 minutes after an exposure of 1 hour in the aerosol chamber revealed striking reproducibility of the census of staphylococci in lungs within each experiment and from experiment to experiment (Tables III and IV).

The rate of disappearance of culturable organisms from normal animals can be seen in Tables III and IV. Although different strains of staphylococci were used in the two experiments, the results are essentially identical. From a 15 minute bacterial census of approximately 40,000 staphylococci per lung (around 4.7 log), clearing rapidly ensued so that after 1 day a 2 log decrease in population had occurred (Table III). This represented the disappearance of approximately 99 per cent of the staphylococci that were present 15 minutes after removal of mice from the aerosol chamber. After 2 days (Table IV), the clearing mechanisms had eliminated 99.9 per cent of the staphylococci originally present and the disappearance of the staphylococci was manifestly complete by the 3rd day.

The fate of the staphylococci introduced by aerosol into the animals infected with influenza virus was apparently identical with the previously described fate of staphylococci when they were introduced by intranasal instillation. As may be seen in Tables III and IV, when staphylococci were introduced after 3 days of viral infection, their number remained constant until the viral infection had evolved through approximately the 7th day. At that time there was once again a rapid disappearance of staphylococci from the lungs.

Incubation and Fixing of Whole Lungs

To determine the localization within the bronchopulmonary tissues of the aerosolized staphylococci, whole lungs were removed immediately after exposure, frozen, and then incubated for 4 hours. The lungs were then fixed in formaldehyde, sectioned, and stained with Gram's stain. These sections clearly demonstrated colonies of staphylococci in the alveolar spaces and terminal bronchioles, as well as in the remainder of the bronchial tree.

TABLE III

Numbers (Log 10) of Culturable Staphylococci (Giorgio Strain) and P. pseudotuberculosis (Figures in Parentheses) Isolated from Lung Homogenates of Mice Exposed to Aerosolized Staphylococci 3 Days after Inoculation of Influenza Virus or Saline

	Time after exposure to staph. aerosol					
	15 min.	1 day	3 days	6 days	9 days	16 days
Aerosol staph., no influenza	4.82	2.96	0 (4.70)	1.78	4.84	0 (2.02)
	4.72	2.88	0 (4.65)	0	0.74	0
	4.68	2.72	0 (4.47)	0	0.74	
	4.65	2.49	0 (4.05)	0	0 (3.00)	
	4.58	2.28	0 (3.58)	0	0	
Aerosol staph. 3 days after influenza		6.15	5.05	1.77	7.09 (8.17)	0.74
		4.91	5.03	1.77	7.01 (6.45)	0 (2.77)
		4.56	4.73	1.63	0.73	0 (0.73)
		4.24	4.25	0	0 (1.03)	0 × 9
		4.15	3.03 (6.36)	0	0	

Introduction of Staphylococci by the Intravenous Route

In previous studies (13, 14) in which the strains of staphylococci used in the present experiments were injected intravenously, it had been observed that an initially high microbial census is attained in the lung and that this census declines steadily but relatively slowly throughout the ensuing 1 or 2 weeks. Accordingly, experiments were performed to study the effect of the influenza virus infection upon the populations of staphylococci in the lungs when the bacteria were introduced by the intravenous route. In Text-figs. 1 and 2 may be seen the results of two intravenous infections with the Giorgio strain of staphylococcus given 3 days after intranasal inoculation of the influenza virus.

For the first experiment 0.2 ml of an 18 hour broth culture of the staphylococci diluted 1:20 was given intravenously. This dose, amounting to 4.8×10^6 staphylococci cells, was less than that customarily employed in the previously studied intravenous infections. The

inoculum was decreased in the present experiment in order to lessen the mortality rate of the injected animals and allow a longer period of survival for study. The usual inoculum, 0.2 ml of a 1:2 dulution of culture, was employed in the second experiment.

The presence of infection with influenza virus of varied duration had no significant influence on the populations of staphylococci in the lungs when the bacteria were introduced by the intravenous route (Text-figs. 1 and 2).

TABLE IV

Numbers (Log 10) of Culturable Staphylococci (Stovall Strain) and P. Pseudotuberculosis (Figures in Parentheses) Isolated from Lung Homogenates of Mice Exposed to Aerosolized Staphylococci 3 Days after Inoculation of Influenza Virus or Saline

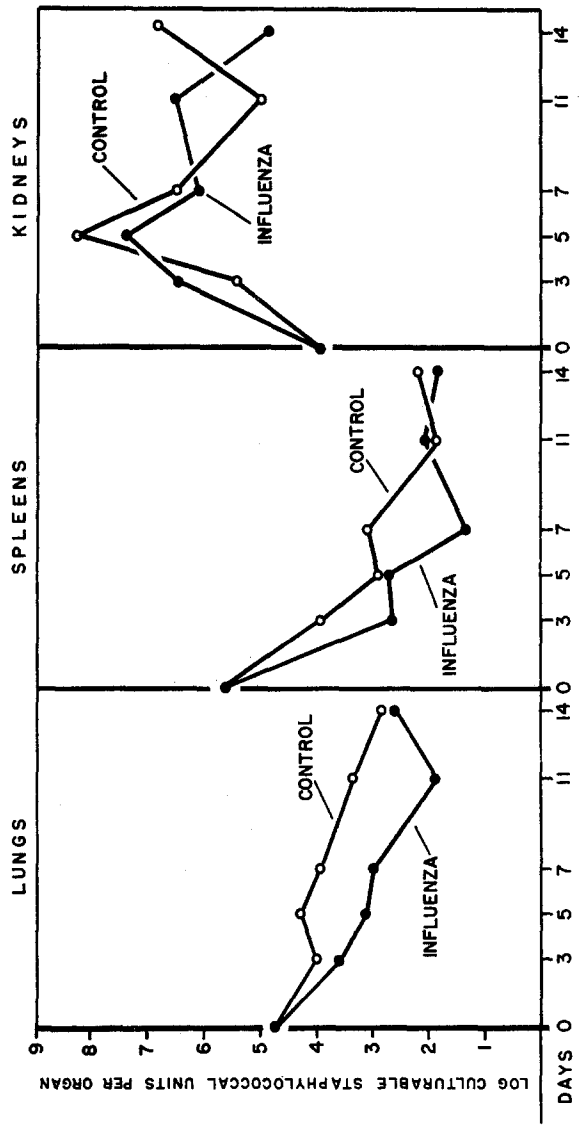
	Time after exposure to staph. aerosol				
	15 min.	2 days	4 days	7 days	9 days
Aerosol staph., no influenza	4.72	1.91	0 (1.33)	0 (6.99)	0
	4.66	1.89	0 (1.04)	0 (3.92)	0
	4.66	1.44	0 (0.75)	0 (3.03)	0
	4.64	1.05	0	0 (1.58)	0
	4.52	1.04	0	0 (1.22)	0
Aerosol staph., 3 days after influenza		3.75	4.43 (8.03)	0.73	0
		3.34	1.44	0 (3.59)	0
		3.33	1.04	0 (3.54)	0
		2.99	0.75 (1.35)	0	0
		2.97	0.73	0	0

The Influence of Prior Influenza Virus Infection Upon the Fate of Intravenously Inoculated Staphylococci in Organs Other Than the Lung

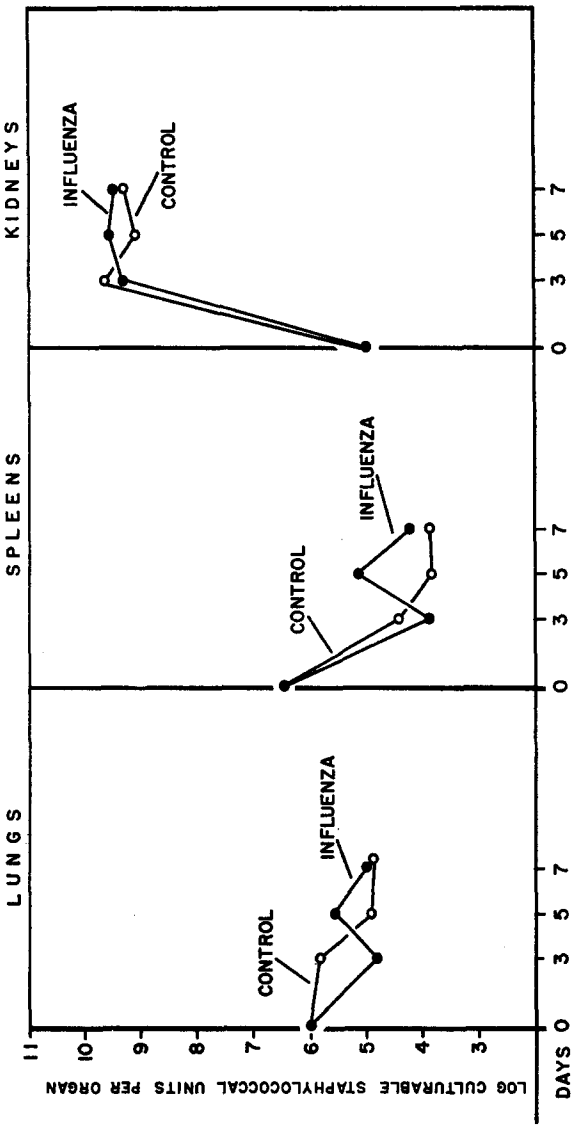
In the experiments described above and illustrated in Text-figs. 1 and 2 populations of viable staphylococci were counted not only in the lungs but also in the spleen and kidney—organs remote from the primary pulmonary site of influenza virus infection. It is notable that in these organs, as in the lung, no significant influence of the virus infection on tissue bacterial counts was evident when staphylococci were injected intravenously. These results are interpreted as evidence against the induction of a general (extrapulmonary) depression of host resistance to staphylococci by influenza virus infection.

Pathological Studies

To determine if the persistence of staphylococci within the bronchopulmonary tissues of mice previously infected with influenza virus had any effect



TEXT-FIG. 1. Fate of intravenous staphylococci in organs of mice infected with influenza virus by intranasal route. Staphylococcal inoculum, 4.8×10^6.



TEXT-FIG. 2. Fate of intravenous staphylococci in organs of mice infected with influenza virus by intranasal route. Staphylococcal inoculum, 6.7×10^7

upon the pathological evolution of the influenza infection, a number of pathological studies were done.

It was clearly established after observations in three different experiments that in the absence of influenza infection staphylococci administered by aerosol produced no demonstrable changes in the microscopic appearance of the lungs. This was true even when the staphylococcal aerosol was given 3 days after saline had been administered intranasally.

Groups of mice infected intranasally with influenza virus and receiving no other challenge were then compared with mice infected with influenza virus and challenged 3 days later with an aerosol of sterile broth or an aerosol of staphylococci. Animals from each group were sacrificed on the day of exposure to aerosol, on the following day, and 3 days later (or 3, 4, and 7 days after infection with influenza virus). Lungs were fixed, sectioned, stained, and examined microscopically. (Data on details concerning the pathological changes in the mouse accompanying infection with the non-adapted strain of influenza virus are being prepared as a separate report by one of the authors, 15.) In general, it may be stated that there was no difference among the three groups. In all three groups there was tracheobronchitis with peribronchial and perivascular infiltration. Lesions tended to progress up to the 7th day and then to diminish gradually. In each group, however, especially on the 7th day after influenza infection, 50 per cent of the animals had more severe lesions. These consisted of necrotic purulent bronchitis, destructive alveolitis with polymorphonuclear cell infiltration, and in some instances abscess formation. There was no difference among the three groups, (influenza and staphylococcal aerosol, influenza and broth aerosol, and influenza alone) in the frequency with which these purulent complications occurred.

Emergence of Endogenous Bacteria Following Influenza Virus Infection

In all of the above studies it was consistently noted that in addition to staphylococci, large numbers of Gram-negative organisms known to be common among mouse colonies were recovered after infection with influenza virus. In the initial studies (see Tables II and III) the superinfection was with *Pasteurella pseudotuberculosis* (as determined by cultural characteristics, fermentation reactions, and staining properties). This organism was found more frequently in the greatest titer in animals infected with influenza of 5 to 10 days previously. Without exception, when large numbers of staphylococci were found in the lung after 7 days of influenza infection had elapsed, they were accompanied by equal or greater numbers of *P. pseudotuberculosis* organisms.

In subsequent studies with different litters of mice a similar percentage of animals with grossly manifest superinfection with Gram-negative bacilli was found, but in these studies *Hemophilus influenzae-murium* was found (see Table V).

It should be noted that in the first studies there were several instances in which control animals received saline intranasally without influenza virus and subsequently were found to have significant numbers of *Pasteurella pseudo-*

tuberculosis in their lungs. In all subsequent studies control animals which received no influenza did not later develop superinfection with Gram-negative bacilli whereas 40 per cent of mice infected with influenza virus subsequently developed superinfection with Gram-negative bacilli.

Effect of Antimicrobial Treatment on the Subsequent Pathogenesis of Influenza Infection in Mice

The frequency of evocation of endogenous microorganisms seemed to correlate well with the frequency of purulent microscopic lung lesions whether or not the animals were secondarily infected with staphylococci. An experiment was designed to determine if the use of antimicrobials to suppress the evocation of infection by endogenous organisms would affect the pathological picture of influenza infection.

Four groups of mice were infected intranasally with influenza virus. Treatment of two of the four groups with penicillin and streptomycin was immediately begun. Initially each mouse received 1.25 mg of aqueous penicillin and 4 mg of streptomycin intramuscularly daily. The untreated groups received saline intramuscularly. On the 2nd day several of the treated animals died following a second injection and therapy was changed to 1.25 mg of penicillin and 2 mg of streptomycin every other day. 3 days after the initiation of influenza infection one of the two treated groups and one of the two untreated groups were exposed to an aerosol of sterile broth. 1, 2, 3, and 4 days later (or 4, 5, 6, and 7 days after the initiation of the influenza infection) animals from all four groups were killed. Lungs were removed with sterile precautions and ground and then streak cultures were made on veal agar plates. The remainder of the ground lung specimens were used for virus titrations. Pulmonary viral concentrations did not vary among the four groups.

Although the bacteriological data were only semiquantitative, several conclusions can be drawn from them (see Table V). The two groups which received antimicrobial therapy were free of culturable bacteria on the final 2 days of the experiment and in the case of the group exposed to an aerosol of broth, no bacteria were cultured on the final 3 days. In none of the 24 observations over the 4 day period were any Gram-negative bacilli found. On the other hand, significant numbers of Gram-negative rods were found in 7 of the 24 animals in the untreated groups.

Pathological Studies on Lungs of Treated and Untreated Groups

On days 4 and 7 of influenza infection, animals were killed from each of the four groups last described. Their lungs were fixed, sectioned, and stained with hematoxylin and eosin. 6 randomized lung sections from each animal were examined for pathological appearance.

Differences were observed between animals that were exposed by aerosol to staphylococci and animals exposed to broth. The most notable differences, however, were seen in the appearance of the lungs of the treated animals as compared to the untreated mice. These differences were most marked on the 7th day after influenza infection.

In treated animals, typical segmental tracheobronchitis with desquamation of bron-

TABLE V

Semiquantitative Estimate of Culturable Staphylococci (Stovall Strain) and of Gram-negative Bacilli in Ground Lung Suspensions of Untreated Mice and of Mice Treated with Antimicrobial Drugs*

All animals challenged on day 0 with influenza virus. On day 3 one-half the animals challenged by exposure to an aerosol of staphylococci.

Aerosol, day 3	Antimicrobial treatment‡	Animal No.	Number of lung colonies, day 4	Animal No.	Number of lung colonies, day 5	Animal No.	Number of lung colonies, day 6	Animal No.	Number of lung colonies, day 7
Staphylococci	—	1	11, staphylococcal	1	9, staphylococcal	1	0	1	0
		2	3, staphylococcal	2	0	2	0	2	0
		3	72, staphylococcal	3	5, staphylococcal	3	0	3	0
Broth	—	1	1, staphylococcal	1	0	1	0	1	0
		2	3, staphylococcal	2	0	2	0	2	0
		3	3, staphylococcal	3	0	3	0	3	0
Staphylococci	0	1	2, staphylococcal	1	5, staphylococcal	1	1, staphylococcal	1	>300 Gram-neg. rod
		2	7, staphylococcal	2	10, staphylococcal	2	0	2	0
		3	82, staphylococcal	3	>300 Gram-neg. rod	3	1, staphylococcal	3	>300 Gram-neg. rod 130, staphylococcal
Broth	0	1	0	1	0	1	0	1	0
		2	0	2	23 Gram-neg. rod	2	1, staphylococcal	2	42 Gram-neg. rod
		3	106 Gram-neg. rod	3	0	3	1, staphylococcal	3	4 Gram-neg. rod

* Numbers and types of colonies isolated on veal agar plates following streaking of one loopful of ground lung suspension.

‡ 1.25 mg penicillin and 2 mg streptomycin intramuscularly every other day.

chial and bronchiolar epithelium, invasion of the submucosa and bronchial musculature by polymorphonuclear cells, and peribronchial proliferation of lymphocytic cells occurred. There was considerable vascular stasis in many instances and frequent evidence of proliferation of interstitial cells. There was no alveolar coating which would resemble hyaline membrane formation. A marked dilatation of the lymphatic spaces and perilymphatic proliferation of round cells was also noted.

The untreated animals, in addition to the above lesions, had a marked purulent reaction in 50 per cent of the lungs examined. There were focal areas of fibrinopurulent bronchopneumonia, extending to abscess formation in three instances. In addition to bronchial desquamation, all of the animals showed bronchial and bronchiolar plugging with fibrin, necrotic cells, and hypersegmented polymorphonuclear cells (see Figs. 1 to 4). Destruction of the basal cellular layer did occur leading to necrosis of the submucosa and typical "false membranes" in three instances. In some animals a genuine thrombo-embolic disease developed and involved both circulations, as demonstrated by sections of the heart in which both mitral and tricuspid valves were found to have necrosis, septic thrombi, and infiltration.

DISCUSSION

The present studies leave no doubt that antecedent infection of mice with influenza A2 virus resulted in abnormal persistence of staphylococci in the lungs when these bacteria were introduced by the respiratory route. That this prolonged survival in the pulmonary tissue was not a consequence of depression of a systemic defense mechanism of the host seems clear when it is noted that the rapid decline in pulmonary staphylococci which follows their inoculation by the intravenous route remained uninfluenced by viral infection of the bronchopulmonary tissues. These disparate results of intravascular and intrabronchial staphylococcal inoculation are not surprising when it is considered that the pathologic effects of infection of mice with "unadapted" influenza virus initially involve the bronchiolar epithelium as shown by the present and other (16) studies. It thus appears likely that the persistence of staphylococci in the lungs of virus-infected animals was a consequence of their delivery to endobronchiolar areas directly damaged by influenza virus. It should be noted, however, that the prolonged inhabitation of the pulmonary tissues by staphylococci did not result in any alteration of the pathological appearance of these tissues in addition to that produced by the introduction of influenza virus alone. Thus, the staphylococci in this situation may be comparable to inert carbon particles which can reflect an altered physiological function of the host, but do not themselves contribute to that alteration (17). These findings are consistent with previous studies of the pathogenicity of staphylococci for mice. Experimental models employing staphylococci in mice require either the intravenous injection of at least 10^6 organisms or intraperitoneal injection with the supplementary use of mucin before progressive infection occurs (18, 19).

The persistent but stable populations of staphylococci in the lungs of animals

infected with influenza virus may be a consequence of a diminished ability of epithelium to destroy or to "wash out" the bacteria with bronchiolar secretions, or alternatively may represent an arithmetic balance of bacterial multiplication and destruction in the damaged tissues.

In the present studies the specific mechanism by which local resistance to staphylococci was impaired during the first 7 days of the influenza virus infection were not identified. The staphylococci inhaled in the presence of viral infection were demonstrably delivered to endobronchiolar sites of epithelial necrosis and fluid transudation. Both these types of tissue environment are of a sort known to hamper phagocytosis. Foci of edema fluid in particular have been shown by Wood and Smith to inhibit the ingestion of staphylococci by polymorphonuclear leukocytes (20). Harford (6) has studied the role of edema fluid produced in the bronchopulmonary tissues of mice by influenza virus and by other techniques not involving the use of an inflammatory agent; the pathogenicity of pneumococci in the mouse lung was greatly enhanced in the presence of edema fluid.

Damage to the cilia of the columnar epithelium of the respiratory tract by the 1957 influenza virus represents another possible mechanism whereby the removal of the inhaled staphylococci might have been hindered. In studies with other influenza virus, however, evidence has been presented that the ciliated epithelium in the mouse respiratory tract was not damaged nor was the function of the cilia demonstrably impaired (21). It seems more likely, therefore, that the interference with the removal of staphylococci noted in the present studies was in some way related to the influence of the virus or the virus-induced inflammatory lesion on the efficiency of the phagocytes, or perhaps merely to the maintenance of a cultural environment favorable for staphylococcal multiplication. The possibility that virus itself may have interfered with phagocytic activity is suggested by experiments with guinea pig phagocytes and influenza virus *in vitro* (22).

The inability of infection with influenza virus to influence the disappearance of staphylococci from extrapulmonary sites is in contrast with the results with other agents. As shown by previous studies with the bacterial enumeration method in mice, certain influences adverse to the host, notably corticosteroid administration, can be readily detected by increases of injected staphylococci within the host's organs (23).

Of great interest was the spontaneous appearance of infections with murine *Hemophilus* and *Pasteurella* in the pulmonary tissues of mice previously infected with influenza virus. Whether or not the animals were secondarily challenged with staphylococci, the superinfection with Gram-negative rods occurred in the same proportion of animals of each group. The two organisms found in different experiments were *Pasteurella pseudotuberculosis* and *Hemophilus influenzae-murium*, both of which have been commonly found in the pulmonary tissue of mice (24). It is worthy of note that in contrast to staphylococci, the

appearance of these organisms in significant numbers in the lungs of mice infected with influenza virus exerted considerable influence on the pathological evolution of the influenza infection. The incidence of purulent bronchopneumonia which at times progressed to abscess formation correlated well with the proportion of animals from which these organisms were cultured in significant numbers from the lungs. The absence of purulent complications in animals which were protected from endogenous infection by the administration of antimicrobial agents, supports the hypothesis that the purulent bronchopneumonia was a consequence of spontaneous secondary infection with these murine bacteria.

It is not possible from the present data to determine if this secondary infection represents the activation of a latent infection already within the mice at the time of their inoculation with influenza virus, or a diminished resistance to exogenous bacteria in the murine environment to which the mice were exposed during the course of the influenza infection.

Arndt (25) has shown that these and other organisms may multiply to high titer from extremely low or non-detectable levels in the lungs within a few hours of the intracutaneous administration of endotoxin, suggesting that these organisms may be present in a "latent" form and were "activated" by the endotoxin.

SUMMARY

Mice infected with a non–mouse-adapted Asian strain of influenza A virus suffered an impaired capacity to destroy or remove staphylococci introduced by the respiratory route. This temporary inhibition of local defense mechanisms was of 7 to 10 days' duration.

The persistence of staphylococci in the lung following influenza did not appear to alter the nature of the pathologic reaction to influenza virus.

The presence of influenza virus infection in the respiratory tract of the mouse did not alter the fate of intravenous staphylococci in the lung or other organs.

In 40 to 50 per cent of mice with influenza, purulent bronchopneumonia and infection with *Pasteurella* and *Hemophilus* of murine origin were noted. A minority of control animals evidenced such infection.

The administration of antimicrobials to which the murine bacteria were susceptible prevented both the appearance of the endogenous infection with *Pasteurella* or *Hemophilus* and the purulent sequelae to influenza virus infection. The true picture of uncomplicated bronchopulmonary influenza virus infection was thus separated from the combined virus-bacteria effect otherwise encountered.

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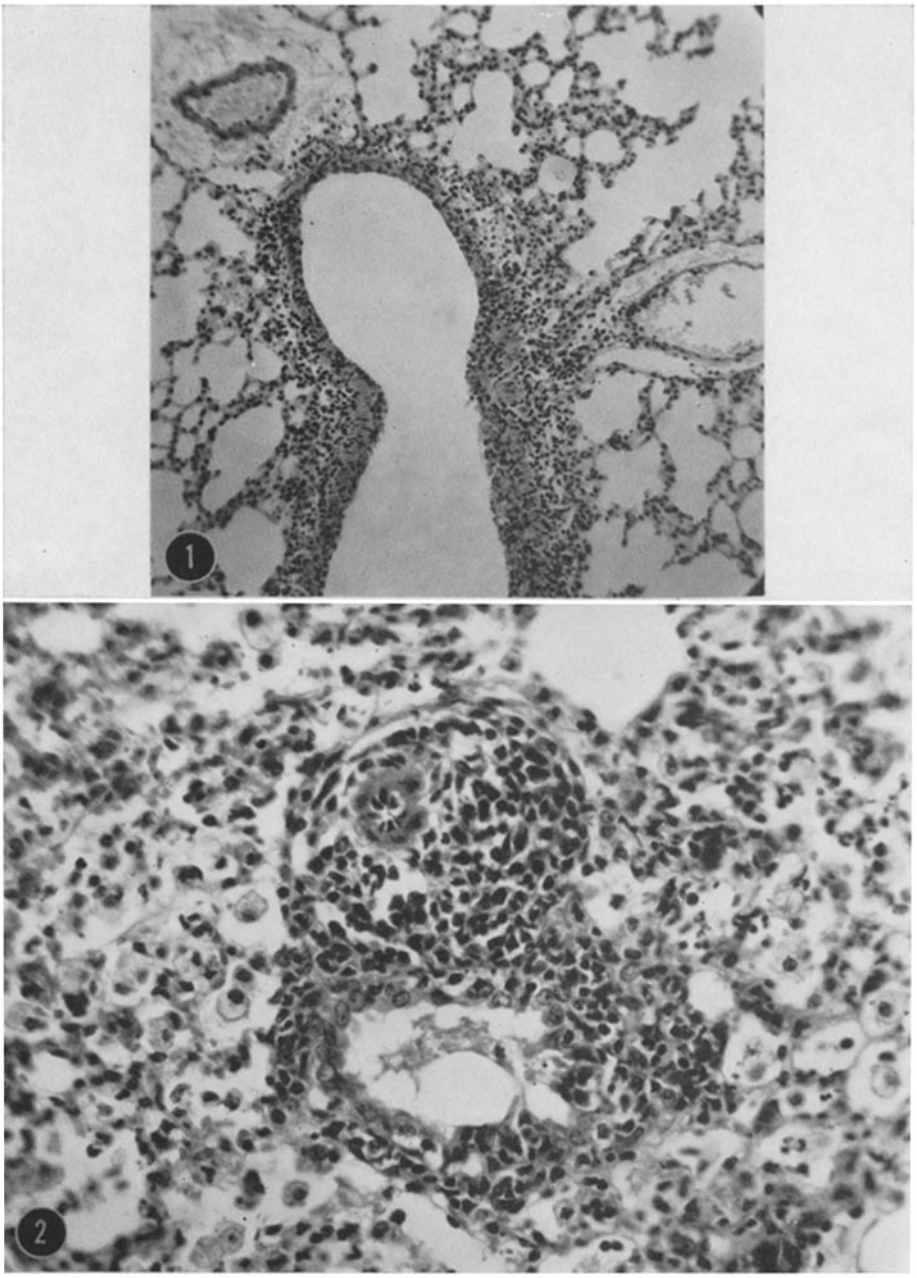
EXPLANATION OF PLATES

PLATE 28

FIG. 1. Influenza infection in mice 7th day of infection. Uncomplicated influenza with bronchial and peribronchial inflammation and perilymphatic round cell infiltration. Magnification × 124.

FIG. 2. Uncomplicated influenza virus infection in mouse (7th day) showing marked perivascular and perilymphatic round cell infiltration. Magnification × 349.

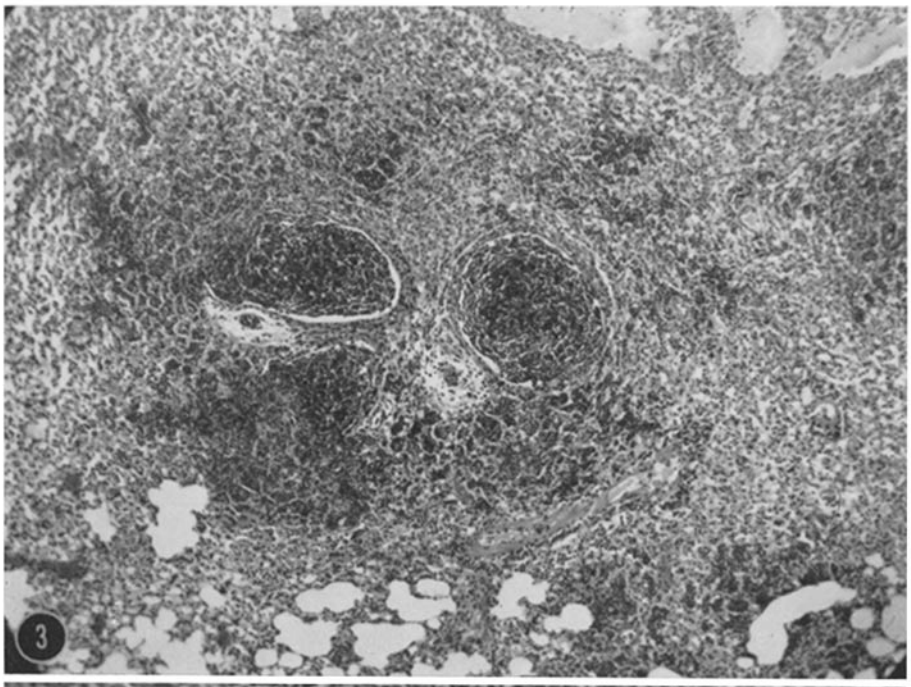


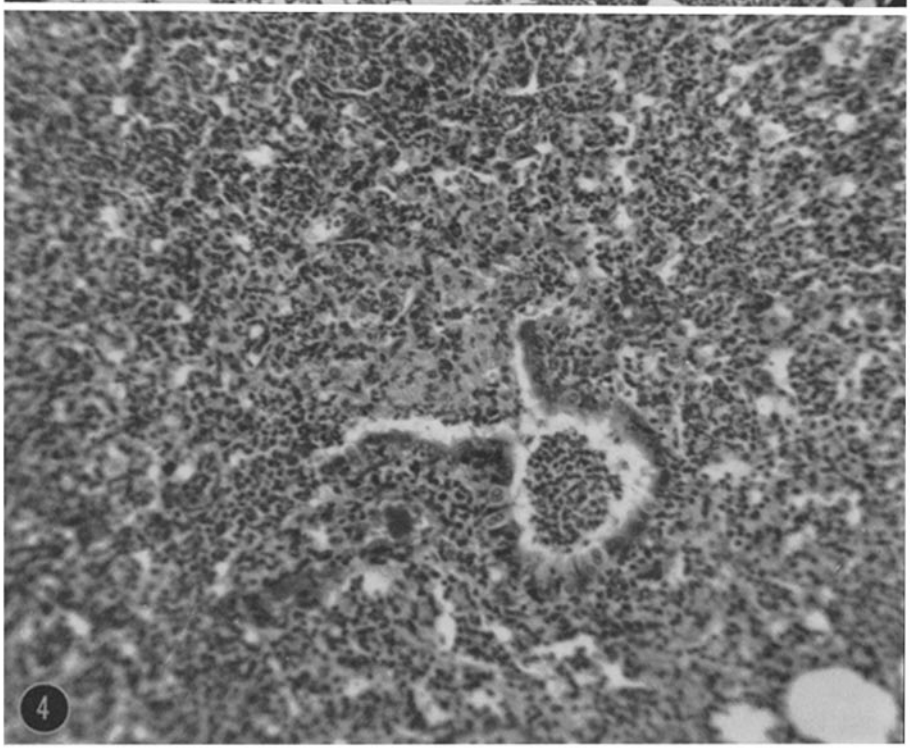
(Sellers *et al.*: Influenza virus)

PLATE 29

FIG. 3. Influenza virus infection (7th day) with bronchial and peribronchial abscesses and patchy areas of fibrinopurulent pneumonia. Magnification × 70.

FIG. 4. Influenza virus infection (7th day) complicated by fibrinopurulent bronchopneumonia with marked polymorphonuclear infiltration. Magnification × 208.





(Sellers *et al.*: Influenza virus)